CHARACTERIZATION OF MAJOR VIRULENCE REGULATORS OF *ERWINIA AMYLOVORA*

BY

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THESIS

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ABSTRACT

Erwinia amylovora is the causal agent of the devastating fire blight disease which is a major concern to the apple and pear industry. Fire blight costs millions of dollars of economic losses all over the world. Exopolysaccharide (EPS) amylovoran and type III secretion system (T3SS) are two major virulence factors in *E. amylovora*. However, how these virulence factors are regulated is not completely understood. In bacteria, gene expression is mainly regulated at the transcription initiation level and its core RNA polymerase (RNAP) requires sigma factors for promoter recognition and initiation. In this study, we investigated the role of several sigma factors in regulating virulence gene expression in *E.amylovora*. Early studies have shown that *hrp*-type III secretion (T3SS) in *E. amylovora* is regulated by HrpS, a member of the σ^{54} enhancer binding proteins, and the master regulator HrpL, which belongs to the ECF subfamily of σ factors. Other sigma factors characterized included RpoN, a nitrogen limitation σ^{54} factor, and its modulation protein YhbH. Our results showed that mutations in *hrp*S, *hrp*L, *rpo*N and *yhb*H resulted in nonpathogenic phenotype in host plant and no hypersensitive response in non-host tobacco. Consistently, expression of T3SS genes including *hrp*L, *dsp*E, *hrp*N and *hrp*A was barely detected in *hrp*S, *hrp*L, *rpo*N and *yhbH* mutants. Amylovoran (EPS) production was higher in these mutants than that of wild type (WT) strain, indicating sigma factors may also play roles in regulating exopolysaccharide production. These results suggest that sigma factors in *E. amylovora* are important virulence regulators and sigma factor cascade exists in its regulatory networks.

Two-component signal transduction systems (TCSTs) in *E. amylovora* play a major role in virulence and in regulating amylovoran production, including EnvZ/OmpR and GrrS/GrrA, two widely distributed systems in gamma-proteobacteria. While both systems negatively control amylovoran biosynthesis, deletion mutants of *envZ/ompR* and *grrA/grrS* have opposite swarming motility phenotypes. In order to determine how the two systems interact, two triple mutants, *envZ/ompR/grrA* (*ERA*) and *envZ/ompR/grrS (ERS*) were generated. Our results showed that both triple mutants had slightly increased virulence on apple shoots as compared to that of wild type (WT) as well as mutants deleting a single system. In an *in vitro* amylovoran assay, amylovoran production was significantly increased in the two triple mutants, indicating the two systems synergistically regulate amylovoran production. In consistent with amylovoran production, *amsG*

gene expression was expressed significantly higher in the triple mutants *in vitro* than those in WT as well as mutants deleting a single system. In contrast, exopolysaccharide levan was significantly reduced in the triple mutants compared with that of WT and deletion of a single system. In addition, the triple mutants showed reduced swarming motility on swarming plates compared to that of *grrA/grrS* mutants and WT strain, but moved slightly faster than that of *envZ/ompR* mutants, indicating that the two systems antagonistically regulate swarming motility in *E. amylovora*. Furthermore, type III secretion (T3SS) genes were significantly upregulated in the triple mutants as well as deletion of a single system than that of the WT strain. These results indicate that EnvZ/OmpR and GrrS/GrrA systems play major roles in virulence and in regulating virulence gene expression**.**

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Literature Review

1.1 Fire blight disease and symptoms

Fire blight is a devastating disease of *Rosaceae* family plants including many economic important fruit trees, such as apple (*Malus sylvestris*) and pear (*Pyrus communis* L.). Fire blight is also the first plant disease attributed to bacterium. The disease is native to North America and the first report of fire blight as a disease of apple and pear occurred in 1780 in the Hudson Valley of New York. Since then, it has spread into every region of the U.S.A. Continually, it was found in some other countries, such as England and New Zealand. By 1990, fire blight was widespread in North America, west Pacific region, Europe and in the Mediterranean area (Bonn & van Zwet, 2000).

The term "fire blight" describes the typical symptoms of this disease as the affected areas turned into black, shrunken and cracked just like scorched by fire. The bacteria are dormant in winter in the infected plant tissues. Open blossoms, tender new shoots, and leaves are the primary infection sites in the spring. Injured tissues are also highly susceptible to infection, including punctures and tears caused by plant-sucking or biting insects. Natural openings, like open stamata, are the entrance sites to cause blackened necrosis, produce viscous exudates and spread throughout the host via vascular system. In some cases, creamy white or yellow ooze droplets containing bacteria and exopolysaccharide are formed at the infected site, which can serve as the source of secondary infection distributed by rain, birds or insects. Abundant moist and heat contribute to disease epidemic: under optimal conditions, this devastating disease can destroy an entire orchard in a single growing season. Over-pruning and over-fertilization can lead to water sprouts and other mid-summer growths that render the tree more susceptible to disease.

1.2 *Erwinia amylovora* and virulence factors

Erwinia amylovora, a highly virulent, necrogenic, vascular pathogen, is the causal agent of disease fire blight. It is a Gram-negative, 0.5-1.0 x 3.0 μm in size, facultative anaerobic, rod shaped bacterium with peritrichous flagella. As a member of the *Enterobacteriaceae*, it is closely

related to *Salmonella entica, E. coli* and *Yersinia pestis.* Two pathogenicity (virulence) factors are strictly required for *E. amylovora* to cause disease: the exopolysaccharide amylovoran and the type III secretion system.

Figure 1.1 Immature pear and shoot with fire blight disease symptoms (Pictures from APSnet)

Recent development of molecular techniques allows the comparison of genome organization of different strains. Great homogeneity was found within *Erwinia* species. Genomic comparison of two *E. amylovora* strains, CFBP1430 (isolated from Crataegus in France) and ATCC 49946 (referred as *Ea*273, isolated from apple in New York), revealed a large-scale chromosomal rearrangement, although they shared more than 99.99% identity at the nucleotide level (Smits *et al.*, 2010; Zhao and Qi, 2011).

Genome sequences of some closely related *Erwinia* species were recently reported and comparative analysis was conducted with genome sequences of *E. amylovora*, *Erwinia pyrifoliae* (isolated from shoot blight in South Korea and Japan), and *Erwinia tasmaniensis* (saprophyte, isolated from trees in UK) (Smits *et al.*, 2011). The presence of several virulence factors, such as T3SS PAI-3, levansucrase, protease A and some effectors, may be responsible for their variance in host range and virulence (Zhao and Qi, 2011). Genetic analysis also revealed that horizontal gene transfer may account for these differential features between the three species (Smits *et al.*, 2011).

1.3 Exopolysaccharide

Exopolysaccharides (EPS) are high-molecular-weight polymers that are composed of sugar residues and are secreted by microorganisms to attach to the cell wall, or secreted into growth medium. EPS is important in biofilm formation and cell attachment to surfaces (Donlan & Costerton, 2002) and is barely immunogenic, which allows pathogens to elude host recognition and escape host defense.

1.3.1 Amylovoran

Like many plant-pathogenic bacteria, *E. amylovora* produces large amount of acidic capsular EPS, called amylovoran. As shown in Fig 1.2, amylovoran is a pentasaccharide repeating units, consisting of galactose, glucose, and pyruvate residues (Nimtz *et al*., 1996).

The amount of amylovoran synthesized can vary for different *E. amylovora* strains. Environmental factors, such as temperature, pH, salt concentration and carbon source, also affect amylovoran biosynthesis. For example, *E. amylovora* are able to synthesis more EPS grown on minimal media with sorbitol compared to grown on nutrient broth–based media (Bellemann *et al.*, 1994).

Amylovoran biosynthetic genes are located within a large 12 gene amylovoran biosynthetic (*ams*) operon, from *ams*A to *ams*L, with *amsG* as the first gene in the operon (Bugert & Geider, 1995). Two genes, *galF* and *gal*E, which are located on the right adjacent to the *ams* cluster, are involved in EPS precursor formation. Products of *ams*G, B, C, D, E, J and K genes play roles in glycosyl transfer for the repeating unit. AmsF seems to be involved in adding newly synthesized repeating units to an existing amylovoran chain. AmsA has tyrosine kinase activity. AmsI was predicted to have a role in recycling of the diphosphorylated lipid carrier after release of the synthesized repeating units (Langlotz *et al*., 2011). Expression of the *ams* operon is regulated by the Rcs phosphorelay two-component regulatory system in *E. amylovora*, which is also essential for pathogenicity (Wang *et al.*, 2009).

Amylovoran is necessary for *E. amylovora* biofilm formation on the surfaces and contribute to pathogenesis, xylem vessel colonization and migration (Koczan *et al.*, 2009). In addition, amylovoran is important in protecting the pathogen from plant defense, and in binding water and nutrients released from damaged plant cells (Leigh & Coplin, 1992). Mutant strains without synthesis of amylovoran were non-pathogenic (Nimtz *et al.*, 1996). The ability of

individual *E. amylovora* strains to produce amylovoran is positively correlated with the degree of virulence (Ayers *et al.*, 1979).

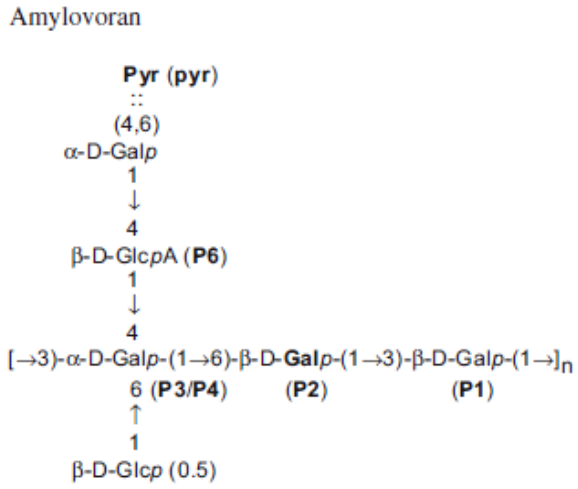


Figure 1.2 The structure of capsular EPS from *Erwinia amylovora*. Glycosyl residues and their linkages in the repeating units of amylovoran are shown. The number 0.5 refers to the glucose residue added to half of the repeating units (Langlotz *et al*., 2011).

1.3.2 Levan

E. amylovora also produces a minor EPS component, levan. Levan is a homo-polymer of fructose residues, the major storage and transport carbohydrates in *Rosaceae* family (Chong & Tapper, 1971). Levan production is controlled by the *lsc* gene, encoding the levansucrase enzyme which is used by *E. amylovora* to cleave sucrose to fructose, then polymerized into levan (Geier & Geider, 1993).

Levan plays a role in *E. amylovora* biofilm formation because a levansucrase-deficient mutant was reduced in biofilm formation and in cell-to-cell aggregation *in vitro* (Koczan *et al.*, 2009). In addition, secretion of levansucrase is thought to contribute to colonization of sucrose-containing tissue by *E. amylovora* (Geier & Geider, 1993) .

1.4 Type III secretion system

Type III secretion system (T3SS) is a protein appendage found in many Gram-negative bacteria, such as *Salmonella, Burkholderia, Yesinia, Pseudomonas, Erwinia, Ralstonia,*

Rhizobium, Vibro and *Xanthomonas.* It is a dedicated mechanism used by bacteria to deliver proteins to cytosol of host cells or apoplast (Galan & Wolf-Watz, 2006; He *et al.*, 2004). In pathogenic bacteria, this needle-like structure is utilized to detect the presence of eukaryotic organisms and secret a variety of effectors across the plant cell wall and plasma membrane in order to assist infection, cause disease symptoms in host plant or elicit hypersensitive response (HR) in non-host plant.

T3SS is one of the most complex secretion systems, which composed of about 30 different proteins (Gophna *et al.*, 2003). A high degree of sequence similarity is observed between T3SS proteins and flagellar proteins (Blocker *et al.*, 2003). The genome sequence reveals three type III secretion systems in *E. amylovora*, including the pathogenicity island 1 (PAI-1) encoded hypersensitive response and pathogenicity T3SS (*hrp*-T3SS), and two *inv/spa*-like non-flagellarT3SS islands (PAI-2 and PAI-3) (Zhao *et al.*, 2009a). Hrp-T3SS has been known for its role as a pathogenicity factor that functions to deliver effectors into eukaryotic host (He *et al.*, 2004). PAI-2 and PAI-3 are similar to SPI1 T3SS of *Salmonella typhimurium* LT-2 and *inv/spa* T3SS of the insect endosymbiont *Sodalis glossidinius str. morsitans*, respectively. However, their functions are still unknown.

1.4.1 T3SS-mediated infection

T3SS is encoded by the *hrp* (hypersensitive response and pathogenicity) genes and *hrc* (*hrp*-conserved) genes among plant pathogenic bacteria (Cornelis & Van, 2000). T3SS proteins can be categorized into three groups, regulatory proteins (e.g. HrpL), secreting proteins (structural components, e.g. HrpA) and secreted proteins (effectors, e.g. HrpN).

T3SS effectors enter into the base of T3SS apparatus and move inside the needle towards the host cell. The detailed mechanism for the entrance of effectors into host cells is still not clear. It is possible that translocators (a set of effectors) are secreted first and form a pore (translocon) in the host cell membrane. Subsequently, other effectors enter into host cells through this translocation pore (He *et al.*, 2004). Mutation in translocator genes didn't affect the secretion of translocator proteins, however, suppressed their ability to deliver them into host cells. Manipulation of host cells by T3SS effectors can be found in several ways: promoting uptake of the bacterium by host cell, tampering with host's cell cycle, inducing apoptosis, or acting as transcription activators.

In *E. amylovora*, *hrc* and *hrp* genes are located in a pathogenicity island (PAI-1) (Oh & Beer, 2005), which also includes *dsp* (disease-specific) genes. Harpin-like proteins (a subset of T3SS substrates) which have the unusual ability to elicit HR, have been reported in all genera of phytopathogens (Ahmad *et al.*, 2001; Alfano and Collmer, 1997). For example, *E. amylovora* and *Pseudomonas syringae* were previously shown to produce two harpins, i.e. HrpN/HrpW and HrpZ/HrpW, respectively (Kim & Beer, 1998).

1.4.2 Regulation of T3SS in *Erwinia amylovora*

In general, T3SS is required by plant pathogenic bacteria for the translocation of certain bacterial proteins to the cytoplasm of plant cell, or secretion of some proteins to the apoplast (Charkowski *et al.*, 1998).

As shown in Fig 1.3, the *hrp/dsp* gene cluster of *E. amylovora* consists of the *hrp/hrc* region and the HEE (Hrp effectors and elicitors) region. The *hrp/hrc* region contains 25 genes, including four regulatory genes, *hrpL, hrpS,* and *hrpXY*, and nine *hrc* genes. The HEE region contains seven genes, such as two harpins genes *hrpN*, *hrpW* and two *dsp* genes (*dspA/E* and *dspB/F*) (Oh & Beer, 2005).

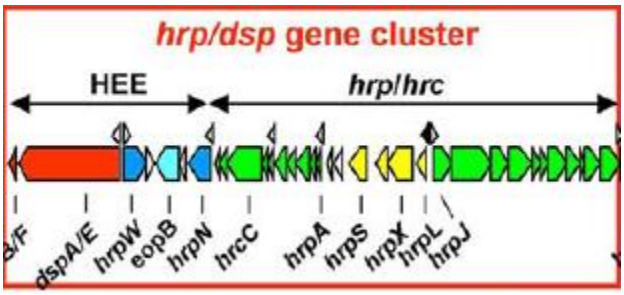


Figure 1.3 The *hrp/dsp* gene cluster of *Erwinia amylovora* strain *Ea*321 *hrp*-T3SS, including the *hrp/hrc* region and the HEE region (Oh & Beer, 2005)

Pathogenicity of *E. amylovora* requires a functional T3SS in which HrpN and DspA/E are found to play an important role in induction of cell death, activation of defense pathways, and ROS accumulation. In *E. amylovora*, HrpL is the master switch of the *hrp* system and belongs to ECF subfamily of σ factors. It controls the expression of five independent *hrp* loci, including *hrp*N, *hrp*W, *hrp*C, *hrp*A and *hrp*J. Wei and Beer proposed that in *E. amylovora*, expression of *hrpL* is environmentally regulated (via HrpX/HrpY two-component regulatory system) and partially controlled by HrpS, which is a member of the sigma 54 factor enhancer binding protein

(Wei & Beer, 1995). However, there is no report as how these factors are involved in regulation of T3SS gene expression.

In general, *hrp* genes are activated in planta, but repressed in rich media. In order to induce T3SS gene expression, minimal media that mimic apoplast conditions is required (Lindgren, 1997), but the conditions can vary considerably between species. For example, *P. syringae hrp* genes are induced at low pH in minimal media, but are nonspecifically repressed by high salt concentrations (Rahme *et al.*, 1992). In contrast, *hrp* genes in *E. amylovora* are stimulated likewise by low pH and repressed by glucose, ammonium salts, asparagine, histidine, and nicotinic acid, however, unaffected by osmolarity (Wei *et al.*, 1992).

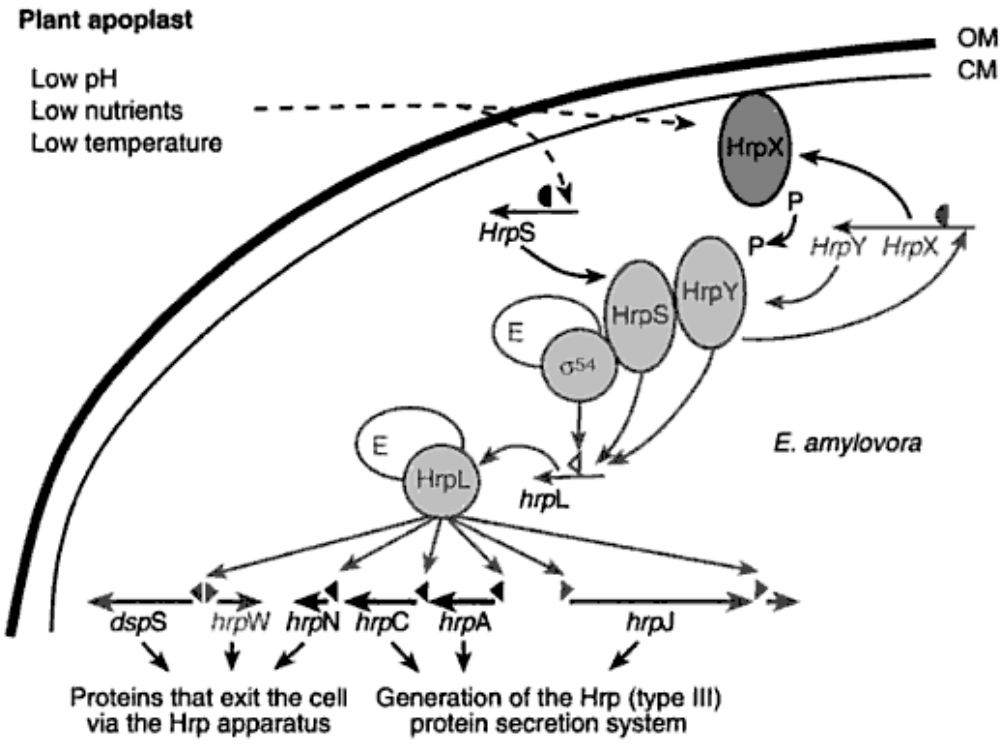


Figure 1.4 Model for the regulation of *hrp* gene expression in *Erwinia amylovora*. Thick arrow lines indicate genes or operons, ovals and circles indicate proteins and arrowheads in thinner lines indicate the directions of information flow. CM, cytoplasmic membrane; OM, outer membrane; P, phosphate; E, RNA polymerase; closed half-circle, σ^{70} promoter; open triangle, σ^{54} promoter; filled triangle, HrpL promoter (Wei & Beer, 1995).

1.5 Sigma factor

In bacteria, gene expression is mainly regulated at the transcription initiation level. Transcription in bacteria is initiated by a PNA polymerase (RNAP) isomerization process in which the promoter DNA is melted close to the transcription start site (Browning & Busby, 2004). Bacterial RNAP holoenzymes are composed of the $\alpha_2\beta\beta'\omega$ core enzyme associated with one of a range of sigma factors. Different sigma factors are activated in response to different environmental conditions. These specialized sigma factors bind to the promoters of genes appropriate to the environmental conditions, increasing the transcription of those genes. There are seven sigma factors in *E. coli,* including the "housekeeping" sigma factor σ^{70} (RpoD), and alternative sigma factors, such as $\sigma^{54/N}$ (RpoN), σ^{38} (RpoS), σ^{32} (RpoH), etc. The primary sigma factor σ^{70} transcribes most genes in growing cells which keeps essential genes and pathways

operating (Gruber & Gross, 2003). Since every molecule of RNA polymerase contains exactly only one sigma factor subunit, there is a competition of RNAP between different sigma factors (Malik *et al.*, 1987).

1.5.1 Classification of sigma factors

Based on their mode of activation, sigma factors can be categorized into two families, sigma 70 family and sigma 54 family. σ^{70} and all alternative sigma factors, except for the homologs of *E. coli* σ^{54}, belong to the extensive sigma 70 family, which directs the binding of RNAP to the consensus −10 (TATAAT) and −35 (TTGACA) sequences to form an open complex to initiate transcription. In contrast, sigma 54 family contains just a single member, σ^{54}, which directs the binding of RNAP to conserved −12 (TGC) and −24 (GG) promoter elements and requires the presence of a specialized activator (bacterial enhancer binding protein) to start transcription (Bush & Dixon, 2012).

1.5.2 RpoN

RpoN ($\sigma^{54/N}$ factor) is the nitrogen-limitation sigma factor that belongs to the sigma 54 family. The range of σ^{N}-dependent genes is still not clear, as the regulated genes described to date control a wide diversity of processes, from flagella, pili to T3SS and EPS production. In general, nitrogen metabolism is regulated by $\sigma^{54/N}$, but many other regulons of $\sigma^{54/N}$ have been identified in several organisms (Kazmierczak *et al.*, 2005).

The σ^{54} has been found to contribute to virulence in a number of Gram-negative bacterial pathogens. In *P. aeruginosa*, *alg*D and *alg*C, two important genes for biosynthesis of alginate (a virulence factor), are controlled by σ^{54} (Peñaloza-Vázquez *et al.*, 2004). Moreover, *rpo*N mutant didn't produce pilin or form pili and had dramatic reduced adhesion (Zielinski *et al.*, 1992). Besides, *rpo*N mutant didn't produce flagellin subunit or form flagella, lost motility, and has attenuated virulence. Similar to *P. aeruginosa*, *Vibrio cholerae* and *Vibrio anguillarum rpo*N mutant lacked flagella and was completely nonmotile. A mutation lacking σ^{54} in fish pathogen *V. anguillarum* severely impaired its ability to infect fish immersed in contaminated water (Damron *et al.*, 2012; Dong & Mekalanos, 2012; O'Toole *et al.*, 1997).

In the case of phytopathogenic bacterial pathogens, RpoN has been implicated indirectly as a regulator of the *hrp* gene cluster. For example, in *P. syringae* pv. *maculicola*, *rpo*N mutant

displayed nonmotile, defected in nitrogen utilization, as well as lost the ability of producing coronatine, causing disease and inducing HR (Hendrickson *et al.*, 2000). RpoN was known to work in conjunction with members of EBPs. In *Pectobacterium carotovora* subsp. *carotovora* strain 71, σ^{54} together with HrpS, one of the NtrC transcriptional activators, are required for activating *hrp*L_{Ecc} transcription (Chatterjee *et al.*, 2002). *P. syringae* RpoN controls *hrp* gene expression and influences virulence via a short regulatory cascade, where HrpR/S activates *hrp*L, and HrpL activates transcription of the remaining *hrp* genes (Grimm *et al.*, 1995). Similar to *P. syringae*, expression of *hrp*L in *E. amylovora* seems to response to various signals and depends on both RpoN and HrpS to activate all *hrp* operons, hrpin and *dsp/avr* genes.

1.5.3 Bacterial enhancer binding protein (bEBP)

Bacterial enhancer binding proteins (bEBPs) are also called σ^{54} activators, which are members of the AAA+ (ATPases associated with various cellular activities) family of proteins that open transcriptional conformation by ATP hydrolysis. The function of AAA+ proteins is converting the chemical energy stored in ATP into a mechanical force that can be used by a series of cellular process (Bush & Dixon, 2012). In the case of bEBPs, they typically bind at -80 to -150 bp upstream of the promoter, which is referred as enhancer sites or upstream activator sequences (UASs) to assist σ^{54} factor.

bEBPs in general consist of three domains, N-terminal regulatory domain, central AAA+ domain and C-terminal DNA binding domain. N-terminal regulatory domain senses signal and modulates the activity of bEBPs; AAA+ domain activates σ^{54}-dependent transcription by providing energy via ATP hydrolysis; C-terminal DNA binding domain contains a helix-turn-helix (HTH) motif, which is responsible for specific UAS site recognition (Bush & Dixon, 2012). The AAA+ domain is the most conserved among the three domains and contains seven conserved regions, including the GAFTGA motif, which forms a loop on the surface of the AAA+ domain to directly contacts σ^{54} during ATP hydrolysis (Bush & Dixon, 2012).

Molecular mechanism of bEBPs in initiating transcription is that at the beginning, six monomers of bEBP form a homohexamer and bind to the UAS site, whereas σ^{54}–RNAP complex binds to the promoter sequence at position −12 (TGC) and −24 (GG), which remains transcriptionally silent. With the assistant of integration host factor (IHF) and DNA looping, σ^{54} directly contacts bEBPs at the conserved motif (GAFTGA). Using the energy provided by ATP

hydrolysis, σ^{54}-RNAP-promoter complex undertakes conformational change, from closed DNA complex to an open one, and thus initiates transcription. Since transcription of a σ^{54} regulated gene can be completely turned on by this mechanism, σ^{54}-dependent gene expression is often responsible for creating swift and precise responses to environmental changes (Kazmierczak *et al.*, 2005).

1.6 Two-component signal transduction systems (TCSTs)

Two-component signal transduction systems are widely distributed in prokaryotes, serving as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to different environmental conditions. In contrast, only a few TCSTs have been reported in eukaryotic organisms.

In bacteria, most TCSTs consist of a membrane-bound sensor kinase (HK) that senses a specific environmental stimulus, and a corresponding response regulator (RR) that mediates the cellular response, mostly through differential expression of target genes. Normally, signal transduction occurs through autophosphorylation reaction (Mascher *et al.*, 2006). After detection of a signal, e.g. a change iron concentration in the medium, two HK monomers dimerize (Stock *et al.*, 2000) and transfer phosphoryl groups from adenosine triphosphate (ATP) to a specific histidine residue in HK. Subsequently, the phosphate groups are transferred to an aspartate residue in the RR. This short phosphorylation cascade causes the conformational change of RR, leading to gene expression (Fig 1.5). The phosphorylation level of the RR controls its regulatory activity (West & Stock, 2001; Stock *et al.*, 1989).

A minority of TCSTs are more sophisticated, which may include a "hybrid kinase", such as the Rcs phosphorelay system. The hybrid kinase consists of not only a kinase domain, but also a receiver domain and an additional phosphorylatable histidine residue, rendering the system to integrate signals into the phosphorelay signaling cascade, and thus can be better fine-tuned (Fig 1.5 bottom) (West & Stock, 2001).

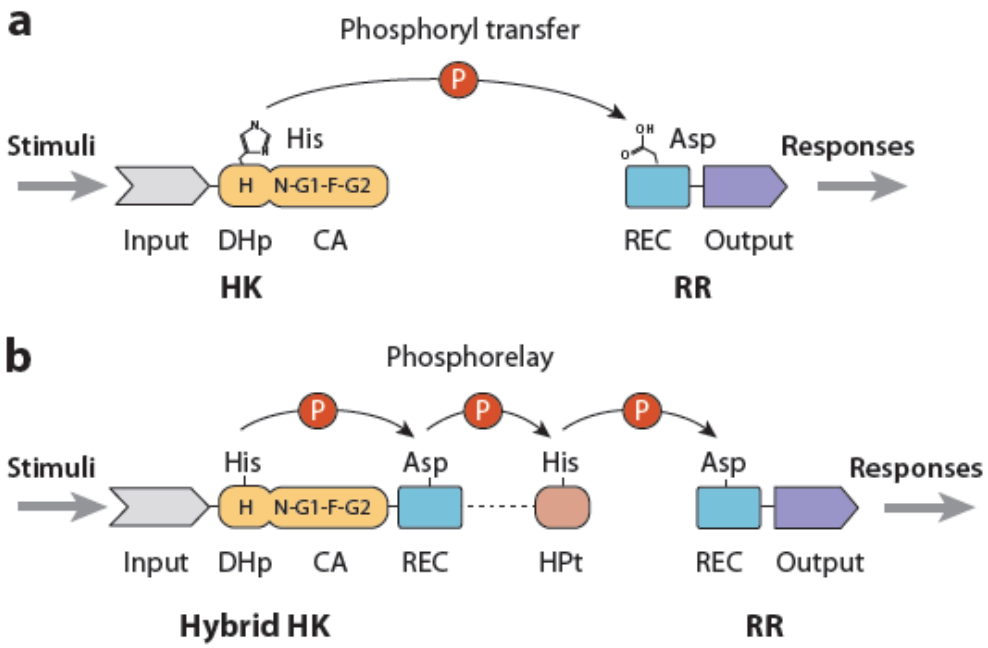


Figure 1.5 Schematic diagrams of two-component signal transduction systems (TCSTs). (a) The prototypical TCSTs pathway features a conserved phosphoryl transfer between the highly conserved kinase core (DHp and CA) and receiver (REC) domains to couple various input stimuli and output responses. (b) A phosphorelay scheme is utilized by hybrid HKs involving additional REC and histidine phosphotransfer (HPt) domains for multiple phosphotransfer events. The intermediate HPt domain can either be an independent protein or linked to the HK (Gao & Stock, 2009).

Given high level of sequence and structural similarity between different systems, more and more people are trying to link together different TCSTs and define their cross-talks or interference, for example, the CpxA-CpxR and EnvZ/OmpR systems in *E. coli* (Siryaporn & Goulian, 2008). It is highly possible that cross-talks between TCSTs enable bacteria to form a small regulatory network to properly react towards environmental changes.

1.7 EnvZ/OmpR and GrrS/GrrA system

Many TCSTs are involved in sensing changes of external environment such as temperature, osmolarity, chemo-attractants and pH. The EnvZ/OmpR and GrrS/GrrA (also called GacS/GacA, BarA/UvrY) are two widely-distributed and well-studied TCSTs in Gamma

proteobacteria. They represent paradigms of signal transduction systems, which have pleiotropic effects, suggesting both systems are global regulators.

1.7.1 EnvZ/OmpR system and its function

EnvZ/OmpR is one of the well-studied TCSTs. It is originally reported to be responsible for osmo-regulation in *E. coli* by governing the expression of *omp*C and *omp*F genes, which encode two major outer-membrane porins, OmpC and OmpF, respectively (Cai & Inouye, 2002). The sensory domain inside HK EnvZ recognizes the variations in membrane surface tension, which triggers conformational changes in EnvZ. At high osmolyte concentration, EnvZ exhibits higher kinase activity than phosphatase activity, resulting in phosphorylation of OmpR. As the number of phosphorylated OmpR proteins increases, OmpR binds to both high affinity binding sites and low affinity-binding repressor site upstream of the *omp*F promoter. OmpR also binds to three low affinity sites upstream of the *omp*C promoter, leading to increased *ompC* expression and thus OmpC becomes the major porin. In low osmolarity state, however, EnvZ exhibits relatively low kinase activity (i.e., high phosphatase activity) towards OmpR, resulting in relatively less phosphorylated OmpR. In this situation, OmpR binds to high-affinity OmpR-binding sites within *omp*F promoter, resulting in OmpF porin production (Kato *et al.,* 1989).

In addition to its role in porin osmoregulation, OmpR has been found to be involved in regulating virulence and various cellular components as a dual regulator, such as EPS synthesis, flagella gene expression, fatty acid transport (Brzostek *et al.*, 2007).

EnvZ/OmpR has been reported to regulate genes associated with virulence in several pathogenic bacteria. The *Shigella flexneri omp*B locus was found to modulate expression of the *vir* genes, which are responsible for invasion of epithelial cells. Mutation of *env*Z gene reduced its virulence (Bernardini *et al.*, 1990). Meanwhile, a mutation in *S. typhimurium omp*R locus resulted in highly attenuated strain (Dorman *et al.,* 1989). OmpR also negatively regulates expression of invasin, a protein that allows enteric bacteria to penetrate cultured mammalian cells in *Yersinia enterocolitica* and T3SS in *P. syringae* (Brzostek *et al.,* 2007). In *Salmonella spp*., OmpR activates another TCST, SsrA/SsrB, which in turn regulates T3SS produced by *Salmonella* pathogenicity island 2 (SPI-2) (Feng *et al.,* 2003). On the contrary, although OmpR was found to be involved in building resistance against phagocytosis or survival within macrophages, mutation in *ompR* did not affect the virulence of *Y. pestis* (Gao *et al.,* 2011).

In addition, EnvZ/OmpR system has been reported to regulate bacterial EPS production. EnvZ/OmpR plays an important role in regulation of Vi polysaccharide synthesis in *S. typhi* and one of the environmental signals for this regulation may be osmolarity (Pickard *et al.*, 1994). The *S. typhi omp*R mutant no longer agglutinates with Vi antiserum. Meanwhile, complementation of the *omp*R mutant with the *omp*R and *env*Z genes of *S. typhi* restores its ability to agglutinate with Vi antiserum. Furthermore, OmpR activates *alg*D in *E. coli*, whose transcription activation is essential for the EPS alginate synthesis and virulence factor expressed by *Pseudomonas aeruginosa* in cystic fibrosis under high osmolarity conditions (Berry *et al.*, 1989).

Swarming is a flagella-driven form of motility for movement across solid surfaces as a group. Hyper-flagellated swarmer cells require EPS and surfactants for mass migration. Inactivation of *omp*R promotes precocious swarming and *flh*DC expression in *E. coli* and *Xenorhabdus nematophila* (Kim *et al*., 2003). In contrast, *Y. enterocolitica omp*R mutant showed a decrease in *flhDC* expression and a non-motile phenotype, suggesting a positive effect of OmpR in regulating flagella master regulator FlhDC (Raczkowska *et al.*, 2011).

A microarray-based comparative transcriptome analysis of *Y. pestis* identified 224 genes whose expression was altered by *omp*R mutation, indicating a global regulatory role in *Y. pestis* (Gao *et al.*, 2011). A similar global regulatory effect of OmpR in *E. coli* was observed (Oshima *et al*., 2002).

1.7.2 GrrS/GrrA system

The hybrid HK GacS (initially called LemA) was first reported in the bean pathogen *P. syringae* pv. *syringae* B728a. The corresponding RR GacA was described shortly thereafter in the biological control bacterium *P. fluorescence* strain CHA0. Subsequently, GrrS and GrrA homologs were identified in many enteric bacteria (*E. coli*, *S. enterica*, *P. carotovora*, *P. fluorescent*, *Vibrio* and *Azotobacter*). The GrrSA system has since been reported to regulate an array of phenotypes, including biofilm formation, alginate biosynthesis, production of toxins and extracellular enzymes, proteases, siderophores, swarming motility and type III secretion system (Zhao *et al*., 2009b). Two main properties of *gac*S/*gac*A mutants stand out: partial or complete reduced biocontrol ability in a group of plant-beneficial *Pseudomonads* and significantly attenuated virulence in plant- or animal-pathogenic bacteria (Altier *et al.*, 2000; Gaffney *et al.*,

1994; Laville *et al.*, 1992; Whistler *et al.*, 1998; Zhang & Normark, 1996). Similar to EnvZ/OmpR, GrrSA also functions as a dual regulator, i.e. positive or negative.

GacS/A controls virulence gene expression in a variety of host-pathogen systems, including *Pseudomonas, Vibrio, E. coli, Salmonella* and *Erwinia*. In most cases, *gac*S/*gac*A mutants showed reduced production of virulence factors and attenuated virulence. In plant pathogen *P. syringae,* GacS/GacA was found to play a role in regulating *hrpRS* expression (Heeb & Haas, 2001). In *D. dadantii*, GacA upregulated *dsp*E, *hrp*A, and *hrp*N *in vitro* and *in vivo* (Yang *et al.*, 2008). The *gac*S and *gac*A mutants of *P. syringae pv. syringae* B728 were completely nonpathogenic in foliar infiltration assays (Willis *et al.*, 2001). Besides, *gac*S and *gac*A mutants of animal pathogens such as *Salmonella spp*. (Johnston *et al.*, 1996), *V. cholera* and *P. aeruginosa* PAO1 (Parkins *et al.*, 2001) were attenuated in colonizing infant mice, suggesting an important role in regulating virulence factors. In APEC (avian pathogenic *E. coli*), GacS/A regulates a variety of virulence factors, such as its abilities to adhere, invade, persist within tissues, survive within macrophages, as well as resistance to serum complement (Herren *et al.*, 2006). In *S. enterica,* GacA (SirA) has been found to contribute to the regulation of T3SS through *hil*A (Ahmer *et al.*, 1999).

Moreover, GacSA regulates EPS production (e.g. alginate) as well as extracellular enzyme production in a variety of species (Cui *et al.*, 2001). In *P. fluorescences* CHA0, GacS/A system tightly controls the expression of antifungal secondary metabolites (e.g. *hcnA*) and extracellular enzymes (e.g. *aprA*) (Heeb & Haas, 2001). The *gac*A gene product of *P. carotovora subsp. carotovora* strain 71 regulates a number of extracellular enzymes (*pel*-1, a pectate lyase gene; *peh*-1, polygalacturonase gene; and *cel*V, a cellulase gene) (Cui *et al.*, 2001).

As a negative regulator, GacSA was also found to down-regulate flagella gene expression of *P. fluorescens* and *E. coli.* Mutation in both *gac*S and *gac*A genes in *P. fluorescens* strain CHA0 affected its motility on swarming plate compared with wild-type strain (Kato *et al.*, 1989). *D. dadantii gac*A mutant showed reduced maceration and systemic invasion ability (Yang *et al.*, 2008). Both *gac*S and *gac*A mutants of *P. syringae* B728a showed reduced ability of swarming (Kinscherf & Willis, 1999). In *S. enterica* serovar *typhimurium,* GacA was found to affect flagella gene expression indirectly by binding to *csrB* promoter and activating its expression (Teplitski *et al.*, 2003).

In addition, GacSA system positively controls the expression of one to five small RNAs (sRNAs), thus upregulates the production of proteins that are otherwise repressed by RNA binding proteins, such as RsmA/CsrA (Cui *et al.*, 2001). In *E. coli*, BarA/UvrY affects the activity of RNA-binding protein CsrA by regulating the expression of *csrB* and *csrC* untranslated regulatory RNA, which bind to CsrA protein and prevents it from binding to target genes (Timmermans & Melderen, 2010).

Recent researches have provided much information that gene expression is mainly regulated at the transcription initiation level in plant- and animal-pathogenic bacterium. *E. amylovora* virulence is controlled by two virulence factors, EPS amylovoran and type III secretion system. However, it is important to note that detailed mechanism of how virulence regulators, such as sigma factors and two-component signal transduction systems are involved in this process is still unclear. Early studies have shown that *hrp*-type III secretion (T3SS) in *E. amylovora* is regulated by the master regulator HrpL, which belongs to the ECF subfamily of σ factors; whereas two-component signal transduction systems (TCSTs) are important regulators of amylovoran production. The main purpose of this project is to identify and characterize major virulence regulators in regulating *Erwinia amylovora* virulence. The specific objectives are:

1. To understand the role of sigma factor 54 and its modulation proteins in *Erwinia amylovora* virulence
2. To determine the interaction between EnvZ/OmpR and GrrS/GrrA systems in regulating *Erwinia amylovora* virulence

Chapter 2

Effect of Sigma Factor RpoN and Its Modulation Protein on *Erwinia amylovora* Virulence

2.1 Abstract

In bacteria, gene expression is mainly regulated at the transcription initiation level and its core RNA polymerase (RNAP) requires sigma factors for promoter recognition and initiation. In this study, we investigated the role of several sigma factors in regulating virulence gene expression in *Erwinia amylovora*, a necrogenic enterobacterium causing fire blight of apples and pears. Early studies have shown that *hrp*-type III secretion (T3SS) in *E. amylovora* is regulated by HrpS, a member of the σ^{54} enhancer binding proteins, and the master regulator HrpL, which belongs to the ECF subfamily of σ factors. Other sigma factors characterized included RpoN, a nitrogen limitation σ factor, and its modulation protein YhbH. Our results showed that mutations in *hrp*S, *hrp*L, *rpo*N and *yhb*H resulted in nonpathogenic phenotype in host plant and no hypersensitive response in non-host tobacco. Consistently, expression of T3SS genes including *hrp*L, *dsp*E, *hrp*N and *hrp*A was barely detected in *hrp*S, *hrp*L, *rpo*N and *yhbH* mutants. Amylovoran (EPS) production was higher in these mutants than that of WT strain, indicating sigma factors may also play roles in regulating exopolysaccharide production. These results suggest that sigma factors in *E. amylovora* are important virulence regulators and sigma factor cascade exists in its regulatory networks.

2.2 Introduction

E. amylovora is the causal agent of fire blight of apples and pears. Its pathogenicity depends on function of hypersensitive response and pathogenicity (*hrp*) type III protein secretion system (T3SS) and production of exopolysaccharide amylovoran (Bellemann & Geider 1992). The regulatory region of the *hrp* gene cluster in *E. amylovora* consists of three adjacent operons: *hrpXY* encodes a two component regulatory system, consisting of histidine kinase (HK) HrpX and response regulator (RR) HrpY; *hrpS* encodes an NtrC-like σ^{54}-dependent enhancer-binding protein; and *hrpL* encodes an ECF (extra cytoplasmic functions) subfamily sigma factor, which belongs to sigma 70 family. The promoter region of *hrpL* contains a putative σ^{54} promoter consensus sequence (Wei & Beer, 1995).

Transcription in bacteria is initiated by a RNA polymerase (RNAP) isomerization process in which the promoter DNA is melted close to the transcription start site (Browning & Busby, 2004). Bacterial RNAP holoenzyme is composed of the $\alpha_2\beta\beta'\omega$ core enzyme associated with one of a range of sigma factors. Every molecule of RNA polymerase contains exactly one sigma factor subunit, so there is a competition of RNAP between different sigma factors. There are mainly two families of sigma factors in bacteria, the sigma 70 family including σ^{70}, σ^{38}, σ^{32}, and σ^{24} (ECF); and the sigma 54 family. Different sigma factors are activated in response to different environmental conditions. σ^{70} (RpoD) is the housekeeping sigma factor that transcribes stringent genes in growing cells and keeps essential genes and pathways operating (Gruber and Gross, 2003). All the other sigma factors are called alternative sigma factors, which competitively bind the promoters of genes under certain environmental conditions. On the other hand, $\sigma^{54/N}$ (RpoN) is the nitrogen-limitation sigma factor whose function requires enhancer binding proteins (EBPs), also are referred to as σ^{54} activators. Typically, EBPs have an N-terminal regulatory domain, a central AAA+ (ATPases Associated with diverse cellular Activities) domain that directly contacts σ^{54} and a C-terminal DNA binding domain (HTH). EBPs usually bind to regulatory DNA sequences upstream from the σ^{54} promoters, from which interact with RNAP associated with σ^{54} (σ^{54}-RNAP) at the promoter.

The mechanisms how sigma 70 and 54 factors work are different. In contrast to σ^{70}–like family sigma factors, which characteristically bind to the -35 (TTGACA) and -10 (TATAAT) positions from the transcription start, σ^{54} family sigma factors bind to specific promoter sequences at positions -24 (GG) and -12 (TGC) and the σ^{54}–RNAP complex forms a closed loop which is transcriptionally silent. EBPs open the transcriptional conformation by ATP hydrolysis within the AAA+ domain, which provides the energy for the conformational change, and thus transcription starts. Since the transcription of a σ^{54} regulated gene can be completely turned on by this mechanism, σ^{54}-dependent gene expression is often counted for creating swift and precise responses to environmental change (Schumacher *et al.*, 2006).

The sigma 54 factors have been indirectly shown to contribute to virulence as a regulator of the *hrp* gene cluster in a number of Gram-negative bacterial pathogens. In *Pseudomonas syringae* pv. *maculicola,* the *rpo*N mutant lost its ability to cause disease and induce HR. In *P. syringae,* expression of *hrpL* was strongly reduced in an *rpoN* mutant (Fellay *et al.*, 1991) and

expression of *Pantoea stewartii hrp* genes was reduced in an *E. coli rpoN* mutant strain (Frederick *et al.*, 1993).

RpoN is also involved in regulating various cellular components, such EPS production and flagella gene expression. In *P. syringae pv. syringae* and *P. aeruginosa*, transcription of *algD* and *algC*, two important genes for biosynthesis of alginate (a virulence factor), are regulated by AlgR and RpoN. Moreover, *rpo*N mutant didn't produce pilin or form pili and had dramatic reduced adhesion (Peñaloza-Vázquez *et al.*, 2004; Zielinski *et al.*, 1992). Besides, the *rpo*N mutant didn't produce flagellin subunit or form flagella, thus losing motility. Similar to *P. aeruginosa*, *Vibrio cholera* and *Vibrio anguillarum rpo*N mutant lacked flagella and were completely non-motile (Damron *et al.*, 2012; Dong *et al.*, 2012; O'Toole *et al.*, 1997). A mutation lacking σ^{54} in fish pathogen *V. anguillarum* severely impaired its ability to infect fish immersed in contaminated water. In the case of *P. syringae* pv. *maculicola*, the *rpo*N mutant was nonmotile and lost its ability to produce coronatine, a phytotoxin (Hendrickson *et al.*, 2000).

In *P. syringae*, two EBPs, HrpR and HrpS are involved in regulating *hrp* T3SS. In this *hrp* regulatory cascade, cooperative action of HrpR, HrpS and RpoN controls *hrpL* gene expression, and HrpL activates transcription of the *hrp* genes (Grimm *et al.*, 1995). In contrast, there is only one EBP HrpS in *E. amylovora* which has been suggested to be required for activating *hrpL* gene expression. HrpL then enables the recognition and transcriptional activation of *hrp* promoters containing "Hrp boxes" in their –10/-35 regions (Fellay *et al.*, 1991).

In the genome of *E. amylovora*, next to *rpoN* is *yhbH* gene (Fig 2.1 A). YhbH is annotated as sigma 54 (RpoN) modulation protein in *E. amylovora*. YhbH, renamed recently as hibernation promoting factor (HPF) in *E. coli*, is involved in ribosome stabilization and preservation in stationary phase by binding specifically to 90S ribosome (a dimer of the 70S ribosomes) to form 100S ribosome. The latter has no translational activity (Kato *et al.*, 2010). However, there is no direct evidence showing exactly how YhbH is involved in sigma 54 regulatory process. On the other hand, both *rpoN* and *hrpS* have not been characterized in *E. amylovora*. The purpose of this study is to systematically characterize the role of RpoN, EBP HrpS and its modulation protein YhbH in *E. amylovora* virulence.

2.3 Materials and methods

2.3.1 Bacterial strains and growth condition

The bacterial strains and plasmids used in this study are summarized in Table 2.1. LB medium is used routinely for culture *E. amylovora*. When necessary, the following antibiotics were added to the medium: 50 µg/ml kanamycin (Km), 100 µg/ml ampicillin (Ap) and 20 µg/ml chloramphenical (Cm). Amylovoran production was determined by growing bacteria in MBMA medium (3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $[NH4]_2SO_4$, 2 ml glycerol, 0.5 g citric acid, 0.03 g $MgSO_4$) amended with 1% sorbitol (Zhao *et al.*, 2009a). A specific *hrp*-inducing minimal medium (HrpMM) containing 20 mmol galactose (1g $[NH_4]_2SO_4$, 0.246 g $MgCl_2 \cdot 6H_2O$, 0.099 g NaCl, 8.708 g K_2HPO_4, 6.804 g KH_2PO_4) was used *in vitro* to mimic conditions of the plant apoplast (Wei *et al.*, 1992).

Table 2.1: Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characters [a]	Reference or source
E. amylovora strains		
Δ*rpoN*	Km^R-insertional mutant of *rpoN* of Ea1189, Km^R	This study
Δ*hrpL*	Km^R-insertional mutant of *hrpL* of Ea1189, Km^R	This study
Δ*hrpS*	Km^R-insertional mutant of *hrpS* of Ea1189, Km^R	This study
Δ*yhbH*	Cm^R-insertional mutant of *yhbH* of Ea1189, Cm^R	This study
E. coli strain		
DH10B	F- *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA1 endA1 ara*Δ139 Δ(*ara, leu*)7697 *galU galK λ – rpsL* (Str^R) *nupG*	Invitrogen, Carlsbad, CA, USA
Plasmids		
pKD46	Ap^R, P_{BAD} *gam bet exo* pSC101 *oriTS*	Datsenko & Wanner, 2000
pKD13	Km^R, FRT *cat* FRT PS1 PS2 oriR6K *rgbN*	Datsenko & Wanner, 2000
pKD3	Cm^R, FRT *cat* FRT PS1 PS2 oriR6K *rgbN*	Datsenko & Wanner, 2000
pGEM® T-easy	Ap^R, PCR cloning vector	Promega, Madison, WI, USA
pWSK29	Ap^R, cloning vector, low copy number	Wang *et al.*, 2011b
pFPV25	Ap^R, GFP based promoter trap vector with a promoterless *gfpmut3a* gene	Valdivia & Falkow, 1997
pBAD30	Ap^R, arabinose-inducing vector	Thao *et al.*, 2010
p*RpoN*	1804bp DNA fragment containing *rpoN* gene in pGEM-Teasy vector	This study
p*YhbH*	2265bp DNA fragment containing *rpoN/yhbH* gene in pGEM-Teasy vector	This study
p*HrpL*	1317bp KpnI-SacI DNA fragment containing *hrpL* gene in pWSK29 vector	This study
p*HrpS*	1810bp KpnI-SacI DNA fragment containing *hrpS* gene in pWSK29 vector	This study
p*HrpL*-BAD	579bp EcoRI-XbaI DNA fragment containing 30 nt 5' of the start codon of *hrpL* gene in pBAD30	This study

a. Km^R =Kanamycin resistance, Ap^R =ampicillin resistance, Cm^R=chloramphenical resistance;

Table 2.2: Primers used in this study

Primer	Sequences (5'—3') [a]	Reference or source
rpoN F	ATGAAGCAAGGTCTACAACTCAGGCTGAGCCAACAGCT TGCCATGACGCCGCGATTGTGTAGGCTGGAGCT	This study
rpoN R	TCAAACCAGCTGTTTACGCTGATTCGATGGCGGGATGG ATAAAGACTCTC ATTCCGGGGATCCGTCGACC	This study
yhbH F	GTTGCATCGTCGACCGACAGCAGGCTTTTTTTGAACAA GGTGAAGAGTTT GCGATTGTGTAGGCTGGAGCT	This study
yhbH R	TAGTTTCACTTACTTATTCACTTCCGCAGGGCGCATGGC ATTTTCCCAGG ATTCCGGGGATCCGTCGACC	This study
hrpL F	ATGACAGAAATTCACCTGCAAACAACTGAATCAACATC GGTCAACGATGGGCGATTGTGTAGGCTGGAGCT	This study
hrpL R	TTAAGAAAATACTGACTGTTTCAGCGTGACGCGCGCAC GCGACAGACGTGATTCCGGGGATCCGTCGACC	This study
hrpS F	AGAGCACATCTCTTTGACAGAAGAACAACCCATCGATA TCCACGACACATGCGATTGTGTAGGCTGGAGCT	This study
hrpS R	GATATAGCGTACGCAAAGGAATACCCAACTCCTGCGCC GCATCATCAATGATTCCGGGGATCCGTCGACC	This study
rpoN Cm1	GTAACAAACTCGCGCAATGG	This study
rpoN Cm2	GCCGATGAACAAGTGAAGC	This study
yhbH Cm1	GTGCCGCGGCTAAAGATTA	This study
yhbH Cm2	TTGTGGCAGGTTAAGCTGTTT	This study
hrpL Cm1	TGCAAATTTTGGCGGTTTA	This study
hrpL Cm2	GCTGGGAAAATTGCATCTC	This study
hrpS Cm1	TGTTCAGCATAAGACGATGG	This study
hrpS Cm2	ATCCCGGCATAACCTTTGTA	This study
Km1	CAGTCATAGCCGAATAGCCT	Zhao *et al.*, 2009a
Km2	CGGTGCCCTGAATGAACTGC	Zhao *et al.*, 2009a
Cm1	TTATACGCAAGGCGACAAGG	Zhao *et al.*, 2009a
Cm2	GATCTTCCGTCACAGGTAGG	Zhao *et al.*, 2009b
16S1	CCTCCAAGTCGACATCGTTT	Wang *et al.*, 2011b
16S2	TGTAGCGGTGAAATGCGTAG	Wang *et al.*, 2011b
amsG-rt1	CAAAGAGGTGCTGGAAGAGG	Wang *et al.*, 2011b
amsG-rt2	GTTCCATAGTTGCGGCAGTT	Wang *et al.*, 2011b
amsD-rt1	GATGCGTCTGTTCAAGCTGT	Wang *et al.*, 2011b
amsD-rt2	TCGCAACAAATCAGTCTGGA	Wang *et al.*, 2011b
rcsA-rt1	TTAAACCTGTCTGTGCGTCA	Wang *et al.*, 2011b

Table 2.2: (Cont.)

Primer	Sequences (5'—3') [a]	Reference or source
rcsA-rt2	AGAAACCGTTTTGGCTTTGA	Wang *et al.*, 2011b
dspE-rt1	TCCAGCGAGGGCATAATACT	Wang *et al.*, 2011b
dspE-rt2	ACAACCGTACCCTGCAAAAC	Wang *et al.*, 2011b
hrpL-rt1	TTAAGGCAATGCCAAACACC	This study
hrpL-rt2	GACGCGTGCATCATTTTATT	This study
hrpN-rt1	GCTTTTGCCCATGATTTGTC	Wang *et al.*, 2011b
hrpN-rt2	CAACCCGTTCTTTCGTCAAT	Wang *et al.*, 2011b
rpoN-rt1/F2	AAGCGGTACTGAAACGGGTA	This study
rpoN-rt2/R2	GCATCAGACTGCGAAAATCA	This study
yhbH-rt1/F3	GCGCGAGTTTGTTACCACTA	This study
yhbH-rt2/R3	ATCGCCGCGTACATATCTTT	This study
hrpS-rt1	AATGCTACGCGTGCTGGAAA	This study
hrpS-rt2	AACAATGGCGTTTGCGTTGC	This study
F1	TCCTGTTGGATGAGCCTTTC	This study
R1	GTACCGTGGGCGATTAAATG	This study
F4	CAGCTTAACCTGCCACAACA	This study
R4	GGCTGGTTATCAATGGCATC	This study
pBAD-*hrp*L-EcoRI	GTCGAATTCGCATCACCTGATTTAGTAACGGAGCAAGCC(EcoRI)	This study
pBAD-*hrp*L-XbaI	TACTTCTAGATTAAGAAAATACTGACTGTTTCAGCGTGC(XbaI)	This study
rpoN co F	CATCTGCAATTTGCGTCACT	This study
rpoN co R	ACCATAACGTCCGTGAAACG	This study
yhbH co F	CGCATCTGCGTGATAGCC	This study
yhbH co R	TTTCCCAGGGTTGGATCATA	This study
hrpL co F	CGGGGTACCTCCTCCATTGAGTCCTCCAG (KpnI)	This study
hrpL co R	AGTAGAGCTCCGACACGCACATGTTCAACA (SacI)	This study
hrpS co F	AGTAGGTACCATGCATGAACGCCTGACG (KpnI)	This study
hrpS co R	AGTAGAGCTCGAATGCGCTCGTCTGTAAGA (SacI)	This study

a. Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primer.

2.3.2 DNA manipulation and bioinformatics analysis

Plasmid DNA purification, PCR amplification of genes, isolation of fragments from agarose gels, cloning, restriction enzyme digestion and T4 DNA ligation were performed using standard molecular procedures (Sambrook & Russell, 1989). Protein domain organizations were derived from the graphical output of the NCBI web interface.

2.3.3 Construction of mutants in *Erwinia amylovora* by Lambda-Red recombinase

E. amylovora stable mutants were generated by using the λ phage recombinases as previously described (Zhao *et al.*, 2009a). Briefly, *E. amylovora* Ea1189 was transformed with plasmid pKD46 expressing recombinases red α, β, and γ. The transformant Ea1189 (pKD46) were grown overnight at 28°C, reinoculated in LB broth containing 0.1% arabinose, and grown to exponential phase OD_{600}=0.8. Cells were collected, made electro-component, and stored at -80°C. Recombination fragments consisting of a kanamycin (*kan*) or chloramphenical (*cat*) gene with its own promoter, flanked by a 50-nucleotide (nt) homology arm, was generated by PCR using plasmid pKD13 or pKD3 as a template. The primers that used for generating mutants are listed in Table 2.2. Primers and internal primer pair Km1 and Km2 of the *kan* gene, Cm1 and Cm2 of the *cat* gene, were used to confirm mutants by PCR. For the resulting mutants, the majority of the coding region of each gene was replaced by the *kan* or *cat* gene, except for the first and last 50 nt. The resulting mutants were designated and listed in Table 2.1.

2.3.4 Virulence assays on apple shoot and immature pear fruit

For *E. amylovora* WT and mutant strains, bacteria were grown overnight in LB broth, harvested by centrifugation, and resuspended in ½*sterile phosphate buffered-saline (PBS) with bacterial cells adjusted to OD_{600} = 0.001 in ½*PBS. Immature fruits of pear (*Pyrus communis* L. cv Bartlett) were surface-sterilized, and pricked with a sterile needle as described previously (Zhao *et al.*, 2006). Two μl of cell suspensions was inoculated on the wounded tissue and incubated the pears in a humidified chamber at 26°C. Symptoms were recorded at 4 and 8 days post-inoculation. For each strain tested, fruits were assayed in triplicate, and each experiment was performed at least three times.

Apple shoot virulence assay was performed on young annual shoots of 'gala' apple, 25 to 40 cm in length. After pricking the tip with a sterilized needle, five µl of pathogen suspension with an initial OD_{600}=0.1 was pipette onto the wounded tissue. For each bacterial strain, seven shoots were inoculated. Plants were kept in a greenhouse at 25°C and a 16 hours light photoperiod, and recorded disease development after 7 days following inoculation by measuring length of the necrotic tissue. The experiment was performed at least three times.

2.3.5 CPC assay for determining amylovoran concentration

Amylovoran concentration in supernatants of bacterial cultures was quantitatively determined by a turbidity assay with cetylpyrimidinium chloride (CPC) as described (Hildebrand *et al.*, 2006). For *E. amylovora* WT and mutants strains, bacterial suspensions was grown overnight in LB broth w/o appropriate antibiotics, harvested by centrifugation and washed with ½*PBS for three times, then resuspended the bacterial pellet in 200 µl PBS and inoculated into 5ml MBMA+1% sorbitol medium with an initial OD_{600}=0.2, inoculated for 24 hours at 28°C with shaking. Following centrifugation, 50 µl CPC at 50 mg /ml were added to one ml supernatant, incubated 10 min at room temperature, and determined amylovoran concentration by measuring OD_{600} turbidity. The final concentration of amylovoran production was normalized for a cell density of 1.0. For each strain tested, the experiment was repeated at least three times.

2.3.6 HR assay

E. amylovora Ea1189 and mutant strains were grown overnight at 28°C. Cells were resuspended to OD_{600} = 0.2 in sterile half-phosphate buffered-saline (½*PBS). When necessary, arabinose was added to bacterial suspension to a final concentration of 0.2%. The mixture was infiltrated into tobacco (*Nicotiana benthamiana*) leaves by needle-less syringe. Infiltrated plants were kept in a humid growth chamber, and HR symptoms were recorded at 24 hours post infiltration. The experiment was repeated at least 3 times.

2.3.7 RNA isolation

For *in vitro* assay, WT and mutant bacterial strains were grown overnight in MBMA+1% sorbitol medium for 24 hours or *hrp*-inducing medium at 18 ºC for 6 hours. For *in vivo* assay, bacterial strains were collected from inoculated pear fruits 18 hour after inoculation as described

above. In both cases, four mL of RNA Protect Reagent (Qiagen) were added to two ml bacterial cultures (at OD_{600} of about 0.5–0.8) to stabilize RNA, harvested the cells by centrifugation for 10 min at 4000 g and extracted RNA using Qiagen Bacterial RNA Mini Kit. Dnase (Qiagen, Hilden, Germany) was used to eliminate residue genomic DNA by an on-column digestion method.

2.3.8 Operon determination

cDNA conversion was performed in a 50-µl reaction mixture by combining five µg of total RNA and 100 ng of random hexamers using the Superscript™ First-Strand Synthesis Kit (Invitrogen). Aliquots of diluted cDNA (2.5 µl, 1:10) were used as template for PCR experiments using 0.3 µM of the required primers and 0.5 µl of Taq DNA Polymerase (Invitrogen). The primer pairs F1 R1, F2 R2, F3 R3 and F4 R4 were used to test their abilities to generate a PCR product for *yhbG, rpoN, yhbH* and *pstN*, respectively (Fig 2.1A). The following primer pairs were used for the transcriptional analysis of the *yhbG-yhbH* operon structure: F1–R2 for the junction between *yhbG* and *rpoN*, F2–R3 for the junction between *rpoN* and *yhbH*, F3–R4 the junction between for *yhbH* and *pstN*. PCR products were analyzed onto 1% agarose gel electrophoresis with ethidium bromide staining (Loisel *et al.*, 2008).

2.3.9 Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed as previously described (Wang *et al.*, 2011) to compare the relative expression of target genes of *E. amylovora rpoN, yhbH, hrpL* and *hrpS* mutants with the WT strain. One microgram of total RNA was reverse-transcribed in a 20 µl reaction using SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). For each sample, negative reverse transcription reaction was done to verify the absence of genomic DNA contamination in subsequent qPCR. Primers (Table 2.2) were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). BLAST searches were performed to confirm gene specificity and the absence of multi-locus matching at the primer site. ABI 7300 System (Applied Biosystems) was used to perform the SYBRGreenq PCR reactions in 96-well optical reaction plates. One µl of cDNA (2 ng/ reaction) or water (no-template control) was used as template for qPCR reactions with Power SYBR Green PCR Master Mix (Applied Biosystems) with a final primer concentration of 500 nmol. Primers in Table 2.2 were used to detect the expression of *E. amylovora amsG, rcsA, dspE, hrpL, hrpS, hrpN, hrpA, rpoN* and *yhbH* gene, respectively. qPCR

amplifications were carried out with a cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, and a final dissociation curve analysis step from 65°C to 95°C. Technical replicate experiments were performed for each biological triplicate sample. Amplification specificity for each qPCR reaction was confirmed by the dissociation curve analysis. Determined Ct values were then exploited for further analysis.

Gene expression levels were analyzed using the relative quantification (ΔΔCt) method. A 16S rRNA rrsA gene was used as the housekeeping gene to normalize our samples ($\Delta Ct = Ct_{target} - Ct_{rssA}$). A relative quantification (RQ) value was calculated as $2^{-(\Delta\Delta Ct = \Delta Ctarget - \Delta Ctreference)}$ for each gene with the control group as a reference. A p-value was computed using a moderated t-test to measure the significance associated with each RQ value. Variations were considered statistically significant when the p-value was <0.05. RQ values for *rpoN*, *yhbH*, *hrpL* and *hrpS* mutants were then normalized to those of WT (Wang *et al.*, 2011).

2.3.10 Statistical analysis

One-way ANOVA and Student-Newman-Keuls test were used determine differences in virulence progress, amylovoran production and gene expression data means within $a = 0.05$, analyzed by SAS 9.2 program.

2.4 Results

2.4.1 Domain organization of HrpL, HrpS, YhbH, and RpoN proteins.

The ECF sigma factor HrpL contains two functional regions (Fig. 2.1B). Sigma70 region 2 is the most conserved part of the protein, which contains both the -10 promoter recognition helix and the primary core RNA polymerase binding site. The other region, sigma70 region 4 is involved in binding to the -35 promoter element via a helix-turn-helix (HTH) motif (Campbell *et al.*, 2002).

Meanwhile, HrpS contains two functional regions, the AAA+ domain that directly contacts σ^{54} and a C-terminal DNA binding HTH domain that binds upstream DNA activator sequences; but lacks the regular *cis*-acting regulatory domain in the N-terminal (Fig. 2.1B). Walker A and Walker B motifs are the most conserved sequences among all the AAA+ members (Schumacher *et al*., 2006) and GAFTGA is the conserved motif that directly interacts with RpoN.

The sigma 54 protein RpoN has three functional regions (Fig. 2.1B). Activator interaction domain (AID) contacts directly with EBPs, thus required for coupling ATP hydrolysis with isomerization of the σ^{54}-RNAP holoenzyme from transcription silent to active states. DNA binding domain (DBD) includes a segment recognizing the -12 promoter region, and a helix-turn-helix motif that specifically interacts with the -24 promoter region. Core RNAP-binding domain (CBD) is a linker between AID and DBD (Hong *et al.*, 2009).

YhbH contains seventeen 30S subunit ribosome binding sites, indicating its ability to attach to 30S subunit of the 90S ribosome in order to form translational silent 100S ribosome.

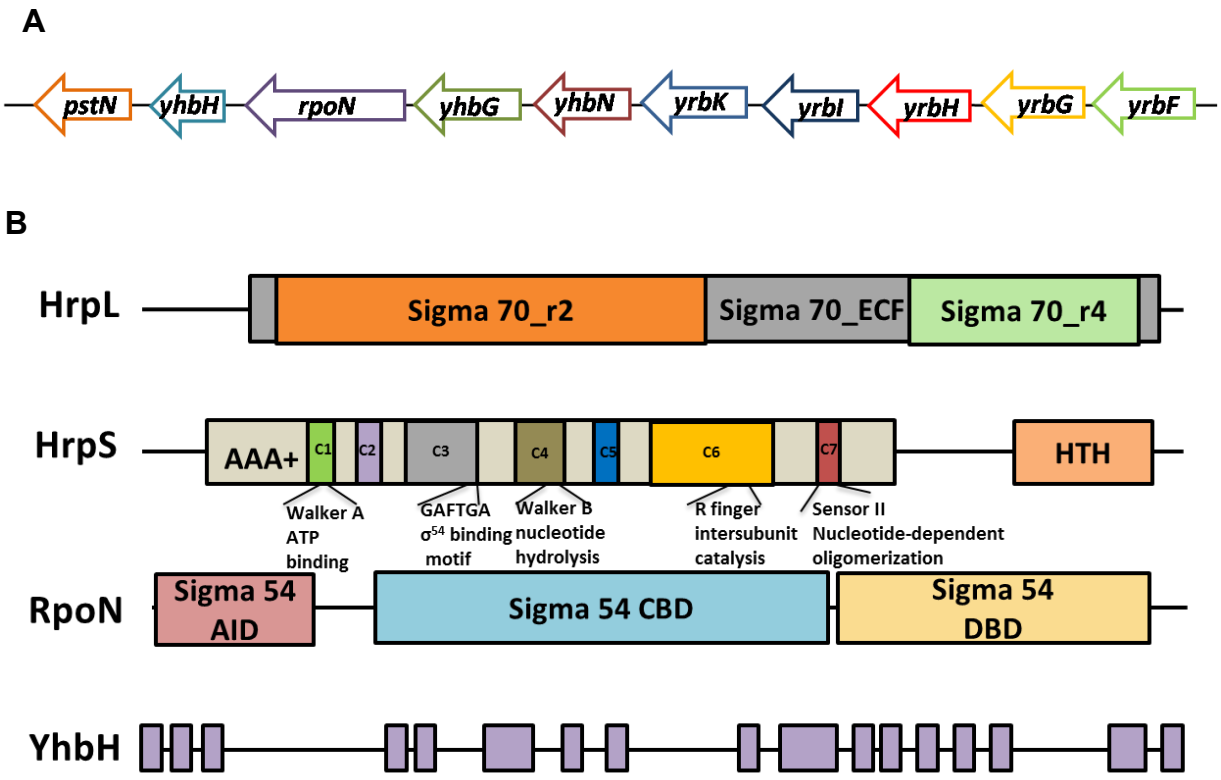


Figure 2.1 Gene organizations and protein domain organizations. A) Gene organizations from *yrbF* to *pstN* gene. Gene arrangement is derived from the NCBI database. B) Domain organizations of HrpL, HrpS, RpoN and YhbH proteins. Domain limits for proteins are derived from the NCBI database. Not drawn to scale. sigma 70_r2, Sigma 70 region 2; ECF, extracytoplasmic function sigma factors; sigma 70_r4: Sigma70 region 4; AAA+, ATPases Associated with diverse cellular activities domain; HTH, helix_turn_helix, DNA binding domain; AID, activator interaction domain, CBD, core binding domain, and DBD, DNA binding domain; ▯, 30S subunit binding site.

2.4.2 *rpoN* and *yhbH* were transcribed under same promoter in an operon

Using operon prediction tool, we have predicted that expression of *rpoN* is under control of promoter of a large operon (from *yrbF* to *yhbH*). It also has been reported that *rpoN* contains its own promoter for its independent expression in *E. coli* (Sperandeo *et al.*, 2006). It has been predicted that in *E. coli*, *yrbK*, *yhbN* (two genes ahead of *yhbG*) transcribed together with *yhbG* (Sperandeo *et al.*, 2006). To determine whether *rpoN* and *yhbH* were transcribed from a single

promoter, PCR amplifications were performed on DNA and cDNA derived from RNA extracted from *E. amylovora* WT. Primer pairs spanning the junction of each gene was used to determine whether two adjunct genes were co-transcribed. As shown in Fig 2.2, no amplification product was detected between *yhbH* and *pstN*. For all other genes, products having expected sizes were amplified, allowing the reconstitution of a continuous 1967-nucleotide-long transcript encompassing *yhbG* to *yhbH* (Fig 2.2, grey arrows). Genomic DNA was used as the positive control and the same PCR mix without the template cDNA cannot amplify the desired product (data not shown). This transcript encodes for the following predicted proteins: YhbG, sigma factor RpoN and sigma 54 protein modulator YhbH.

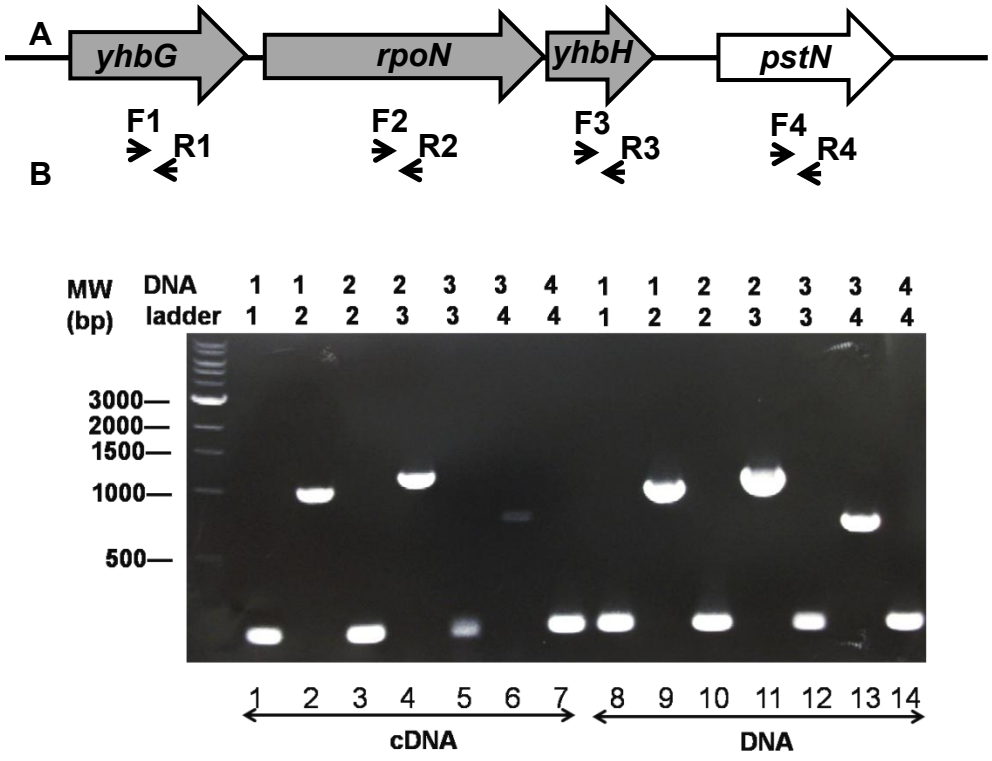


Figure 2.2 Boundaries of the operon. A. Diagram of the analyzed locus from *yhb*G to *pst*N. Primer pairs were localized in each gene. The identified operon was symbolized with grey arrows. B. Agarose gel electrophoresis of the PCR products. Two adjacent genes were declared in an operon when a PCR product was detected with primer pairs spanning the junction of each gene. Correct size of the amplicons was checked by the mean of the DNA ladder shown in the first lane (Invitrogen).

2.4.3 Mutations in *rpoN, yhbH, hrp* and *hrpS* render *Erwinia amylovora* nonpathogenic

In order to characterize the effect of sigma factors RpoN and HrpL, EBP HrpS, and sigma 54 modulator protein YhbH on *E. amylovora* virulence, four mutants were generated and tested for virulence on immature pear fruits. For the WT strain, after 2 days, water soaking showed up at the point of inoculation; after 4 days, necrosis lesion turned black, with visible ooze formation; after 6 days, necrotic tissue enlarged with ooze; after 8 days, blacken necrotic areas covered almost the whole pear fruits with more ooze production. However, for *rpoN, yhbH, hrpL* and *hrpS* mutant strains, no symptom was observed (Fig 2.3 top). These results indicate that RpoN, YhbH, HrpL and HrpS are required for *E. amylovora* to cause disease.

In order to make sure that the absence of disease symptoms of the four mutants was caused by mutations in the *rpoN, yhbH, hrpL* and *hrpS* genes, we transformed the four mutants with plasmids containing the *rpoN, yhbH, hrpL* and *hrpS* genes, respectively. Four complementation strains produced comparable disease progress and disease symptoms as the WT strain on immature pear fruits (Fig 2.3 bottom).

In addition, virulence assay on "gala" apple shoots was also performed for mutants and their complementation strains. The WT strain caused typical "shepherd's crook" symptoms and visible necrosis (Fig 2.4 B). No symptom was observed on apple shoots inoculated with *rpoN, yhbH, hprL* or *hrpS* mutants, as well as the negative control. Consistently, complementation strains restored virulence of *rpoN, yhbH, hrpL* and *hrpS* mutants with comparable disease progress as the WT strain. These results indicate that HrpL, HrpS, sigma factor RpoN, and its modulation protein YhbH are required for virulence.

2.4.4 Mutations in *rpoN, yhbH, hrpL* and *hrpS* abolish *Erwinia amylovora* abilities to elicit HR

In *E. amylovora*, HR inducing activity requires a functional T3SS, especially the translocation of harpin HrpN (Wei *et al.*, 1992). All four mutants and their complementation strains together with WT strain were tested for their ability to elicit HR on tobacco. As expected, *rpoN, yhbH, hrpL* and *hrpS* mutants were unable to cause HR on tobacco, meanwhile normal tissue collapse was observed for all complementation strains as well as WT after 24 h (Fig. 2.5).

These findings demonstrated that HrpL, HrpS, sigma factor RpoN and its modulation protein YhbH are required to elicit HR on non-host tobacco.

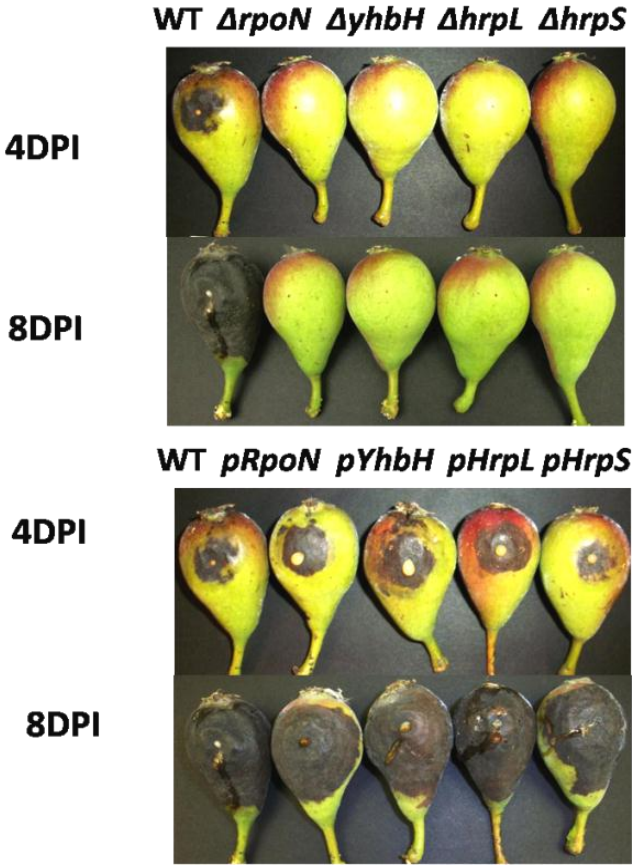


Figure 2.3 Virulence assay for WT and mutant strains of *Erwinia amylovora* on immature pear fruits. Pictures were taken 4 and 8 days post inoculation. Immature pear fruits were inoculated with 2µl of bacterial suspension (OD_{600}=0.01).

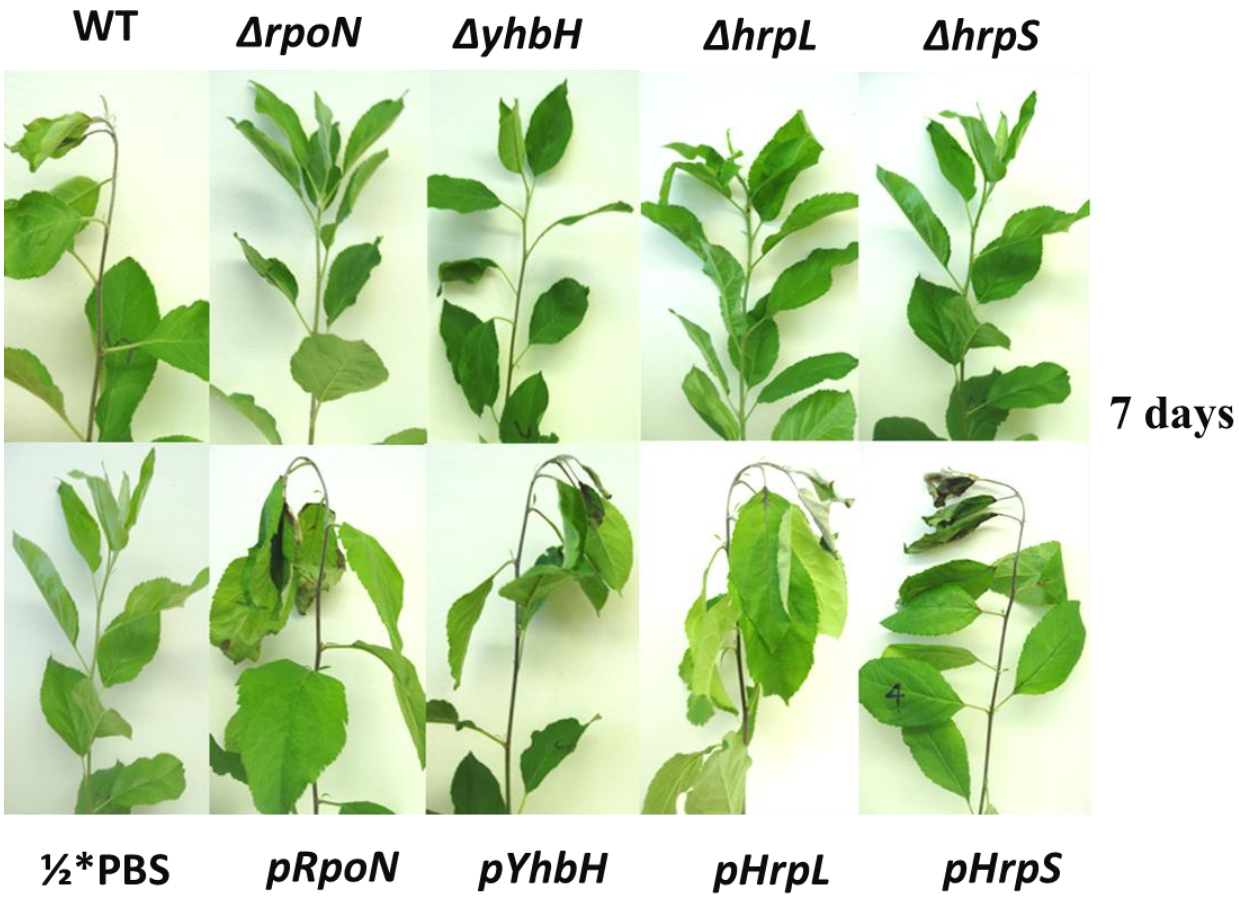


Figure 2.4 Virulence assay for WT, mutants and complementation strains of *Erwinia amylovora* on "gala" apple shoots. "Gala" apple shoots were inoculated with 5µl of bacterial suspension (OD_{600}=0.1). Picture was taken at 7 days post inoculation. For all the strains tested in virulence assay, experiment was repeated at least three times.

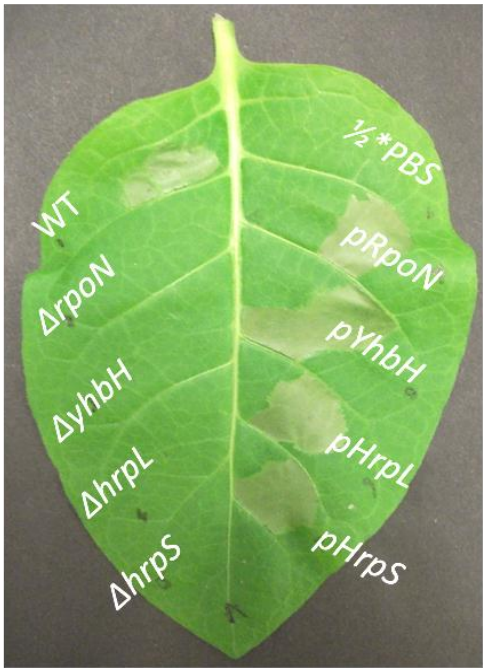


Figure 2.5 HR assay for WT, mutants and complementation strains of *Erwinia amylovora* on non-host tobacco. Pictures were taken 24h post inoculation. Tobacco leaves were infiltrated with bacteria suspension (OD_{600}=0.2) using syringe and incubated at 28°C growth chamber for 24 hours.

2.4.5 RpoN, YhbH, HrpL and HrpS suppress amylovoran production *in vitro*

To determine whether sigma factors and its modulation proteins of *E. amylovora* affect amylovoran biosynthesis, bacterial cells were grown in MBMA+1% sorbitol medium and quantified by CPC turbidity assay (Bellemann *et al.*, 1994). *rpoN*, *yhbH*, *hrpL* and *hrpS* mutants produced higher amount of amylovoran than that of WT at 24 hours post inoculation (OD_{600}=0.08), indicating a negative effect on EPS production (Fig 2.6). Introduction of original copy of *rpoN* and *yhbH* gene into the *rpoN* and *yhbH* mutant strains can partially restore the amylovoran synthesis, producing approximately 0.3 and 0.5 times of amylovoran as the mutants, respectively. These results suggest that RpoN and YhbH of *E. amylovora* are negative regulators of amylovoran production *in vitro*.

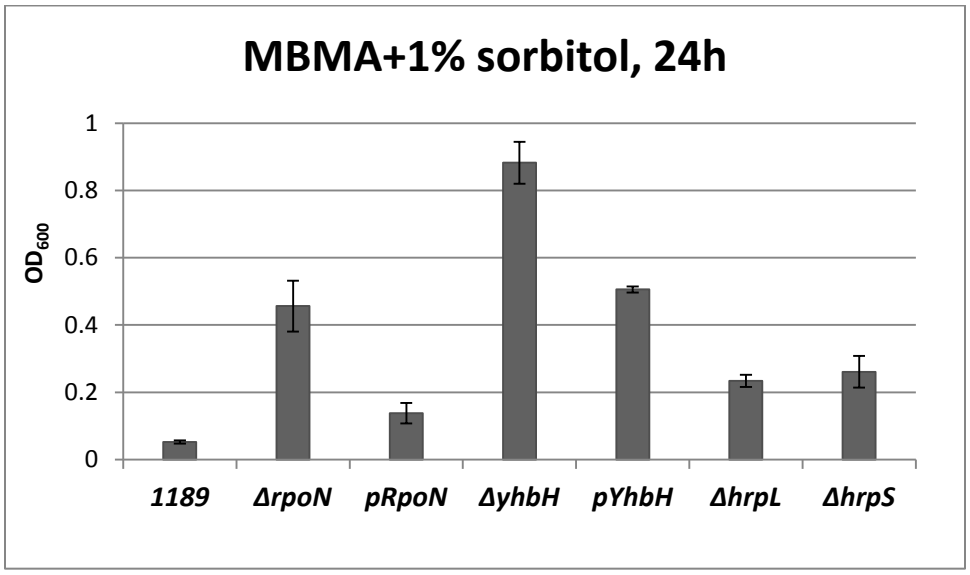


Figure 2.6 EPS amylovoran production of *Erwinia amylovora* WT, mutant and complementation strains. Amylovoran was measured 24h post inoculation in MBMA + 1% sorbitol medium.

2.4.6 RpoN, YhbH, HrpL and HrpS regulate expression of amylovoran biosynthetic and T3SS genes *in vitro*

To correlated amylovoran production with amylovoran biosynthesis gene expression, the expression of the first gene in the amylovoran operon, *ams*G, and the amylovoran biosynthesis rate-limiting regulatory gene, *rcs*A, were quantified by qRT-PCR. mRNA were isolated from bacterial cells grown in MBMA+1% sorbitol medium for 24 h, followed by reverse transcription. Expression of *amsG* and *rcsA* genes was about 10- to 15-fold higher in *rpoN* mutant, 15-fold higher in *yhbH* mutant and 5-fold higher in *hrpL* mutant compared with WT (Fig 2.7A); while their expressions in *hrpS* mutant was about 0.9- and 1.3- fold, respectively.

T3SS gene expression was also examined by qRT-PCR using mRNA isolated from bacterial cells grown in *hrp*-inducing medium for 6 hours at 18ºC. As expected, no expression of *rpoN*, *yhbH*, *hrpL* and *hrpS* gene was detected in the corresponding mutants, respectively (Fig 2.7 B). Consistent with the absence of disease symptoms on host plant, T3SS genes including *dspE*, *hrpL*, *hrpN* and *hrpA* were basically not expressed in all four mutants (Fig 2.7 B). Interestingly, *hrpS* gene was expressed at the same level in *rpoN*, *yhbH* and *hrpL* mutants as compared with WT. Similarly, expression of *rpoN* gene was not affected in the *yhbH*, *hrpL* and

hrpS mutants. Meanwhile, expression of *yhbH* was about two-fold lower in the *rpoN* mutant than that in the WT, but no change was detected in *hrpL* and *hrpS* mutants. These results showed that expression of T3SS-related genes required functional expression of *rpoN*, *hrpL*, *hrpS* and *yhbH* genes. Losing any of these four genes would disrupt the normal operation of *hrp*-T3SS apparatus. In addition, expression of *rpoN* and *hrpS* seemed independent from each other.

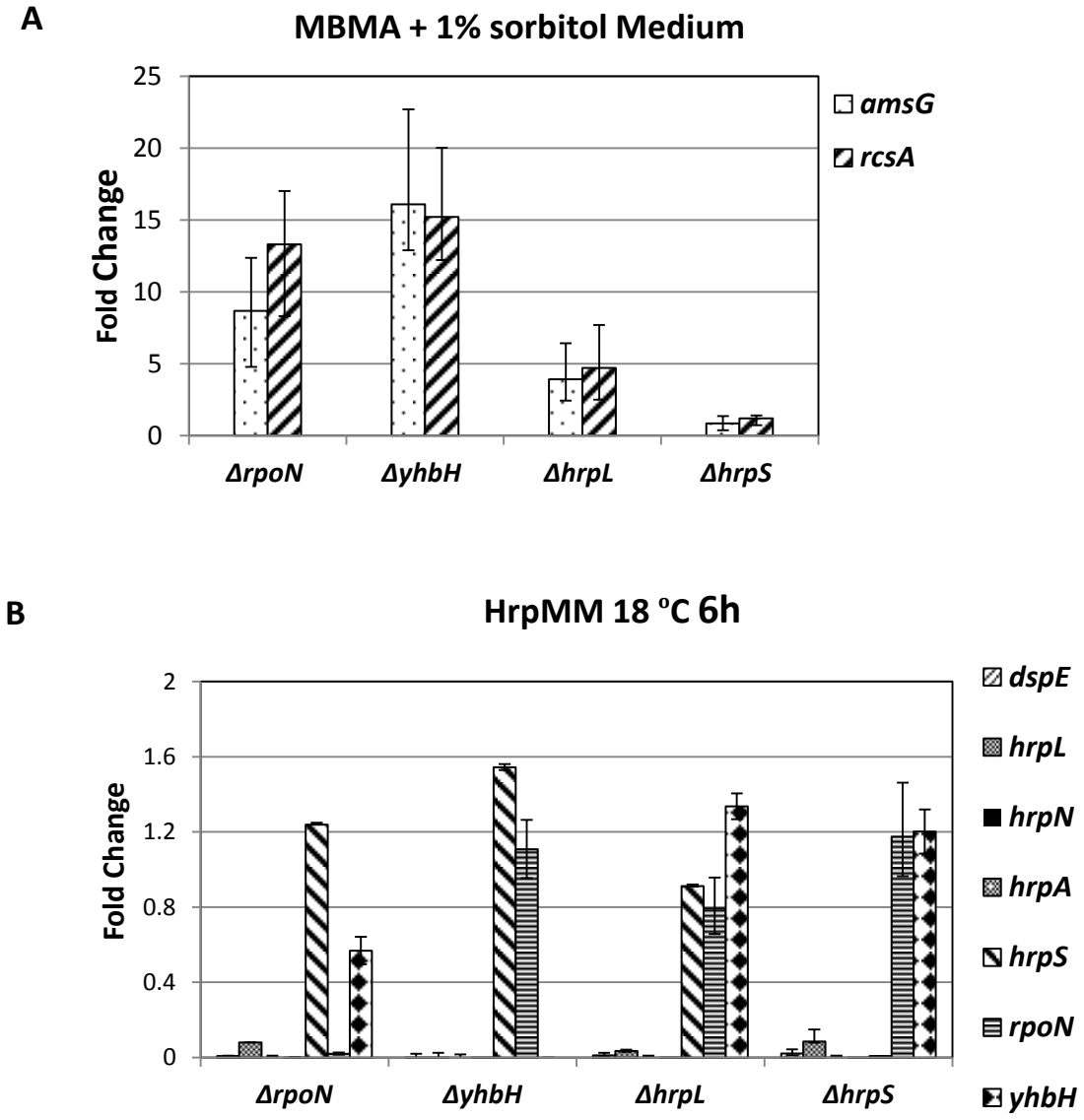


Figure 2.7 Gene expression of selected genes determined by qRT-PCR *in vitro*. The relative fold change of each gene was derived from the comparison of mutant versus wild type in MBMA + 1% sorbitol medium (A), and *hrp*-inducing minimal medium (B). 16S rRNA gene was used as endogenous controls.

2.4.7 RpoN, YhbH, HrpL and HrpS regulate T3SS gene expression *in vivo*

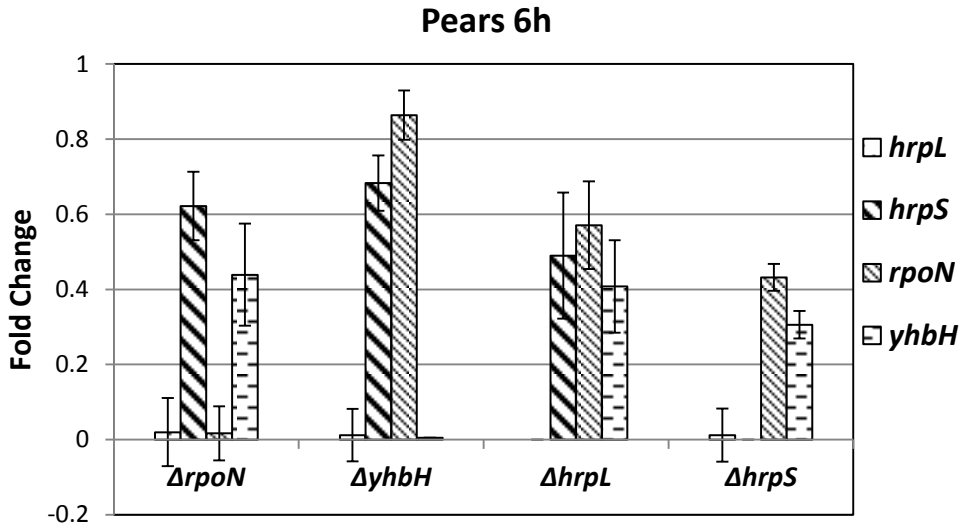


Figure 2.8 Gene expression of selected genes by qRT-PCR *in vivo*. The relative fold change of each gene was derived from the comparison of mutant versus wild type on immature pear fruit. 16S rRNA gene was used as endogenous controls.

To verify the expression of T3SS regulatory genes, *in vivo* gene expression experiment was also performed. As shown in Figure 2.8, the trends in gene expression were the same as *in vitro* data. No expression of *rpoN*, *yhbH*, *hrpL* and *hrpS* gene was detected in the *rpoN*, *yhbH*, *hrpL* and *hrpS* mutants, respectively (Fig 2.8). Consistently, expression of *hrpS* in the *rpoN* mutant was comparable to WT, but *yhbH* expression in the *rpoN* mutant was about half of that in WT strain. Similarly, neither *rpoN* nor *hrpS* expression in the *yhbH* mutant was affected. In the *hrpL* mutant, expression of the other three genes was not significantly different from those of WT. However, *rpoN* and *yhbH* gene expression in the *hrpS* mutant was reduced about two times as compared with WT strain, respectively. These data demonstrated that RpoN, YhbH, HrpS and HrpL are absolutely required for the functional expression of *hrp*-T3SS genes.

2.4.8 Over-expression of *hrpL* restores HR in *rpoN, yhbH* and *hrpS* mutants

Based on gene expression data, it appears that *rpoN* together with *hrpS* and *yhbH* regulates *hrpL* and other T3SS gene expression. Over-expression of *hrpL* would restore HR-inducing phenotypes of *rpoN*, *yhbH* and *hrpS* mutants. *hrpL* gene was cloned into plasmid

pBAD30 with arabinose-inducing promoter. The resulting plasmid *pHrpL*-BAD was transformed into *rpoN*, *yhbH*, *hrpL* and *hrpS* mutants and tested for hypersensitive response in tobacco.

As shown in Fig 2.9, all four mutants containing *pHrpL*-BAD restored their abilities to induce HR in tobacco under inducing conditions, but not without induction. These results demonstrated that sigma factor RpoN, its modulation proteins YhbH, and HrpS, functions at the upstream of HrpL to regulate *hrp* gene expression.

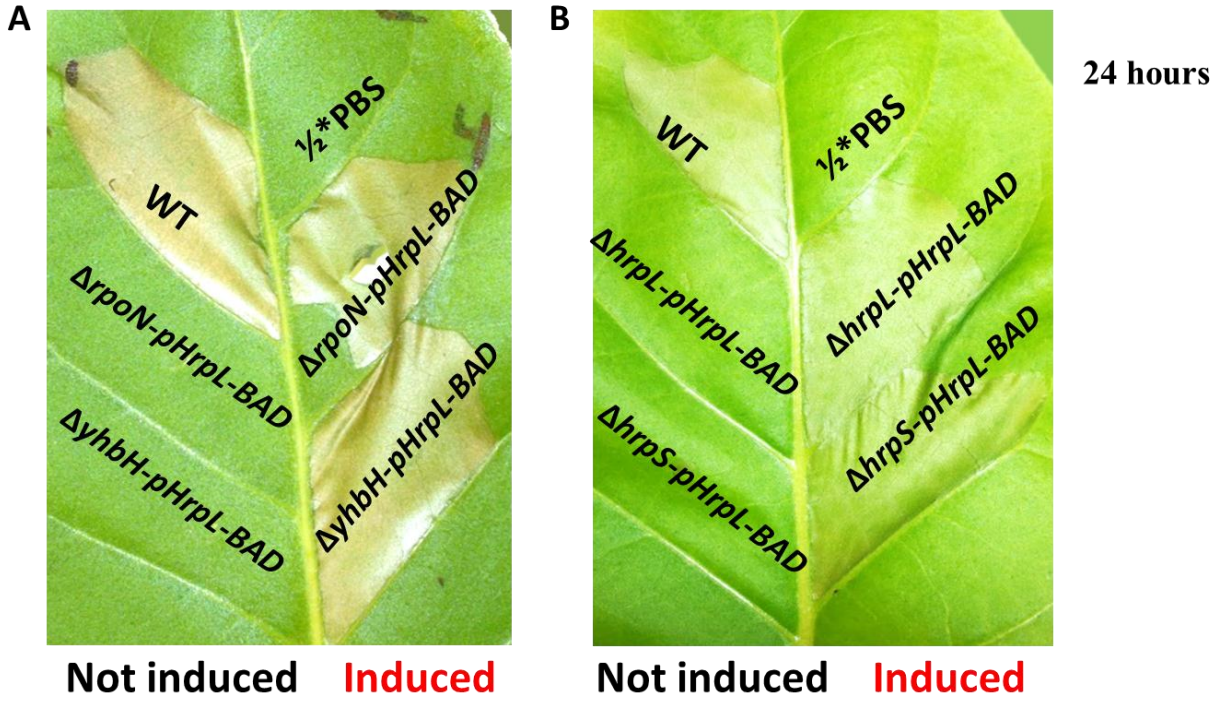


Figure 2.9 Over-expression of *hrpL* restored HR symptoms of *rpoN, yhbH, hrpL* and *hrpS* mutants. *hrpL* gene cloned in an arabinose-inducing vector pBAD30 was introduced into all four mutants. Bacterial suspensions (OD_{600}=0.2) was infiltrated into tobacco leaves with (A and B right) or without (A and B left) 0.2% arabinose. Symptoms were recorded 24 h post inoculation. ½*PBS was used as negative control.

2.5 Discussion

In this study, we investigated the role of sigma factors and its modulation proteins in the regulation of *Erwinia amylovora* virulence. We determined that alternative sigma factors RpoN, ECF sigma factor HrpL, σ^{54} modulation protein YhbH and σ^{54}-dependent EBP HrpS were all required for virulence and expression of *hrp*T3SS genes. It is obvious that a sigma factor cascade exits in *E. amylovora* in regulating T3SS. All four proteins regulate T3SS at the transcriptional level, however, transcription of *rpoN* and *hrpS* is independent from each other.

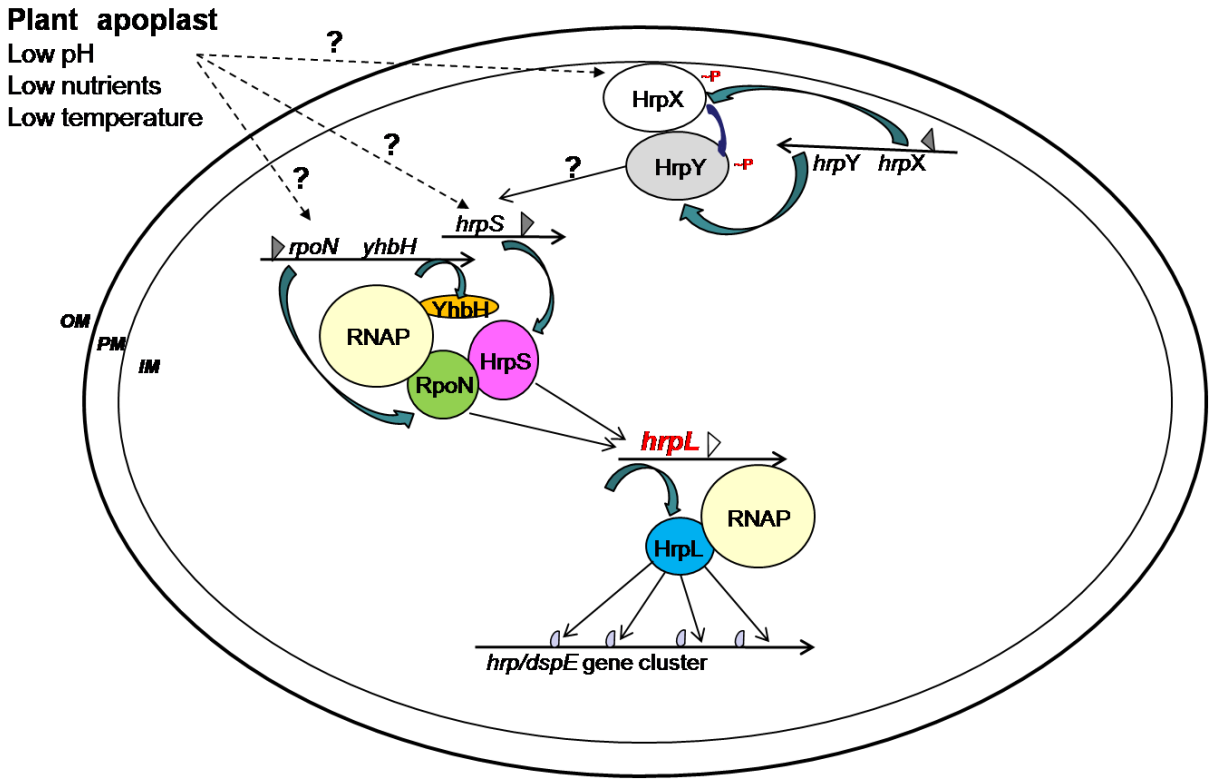


Figure 2.10 Model for the regulation of *hrp* gene expression in *Erwinia amylovora*. HrpX/HrpY is the two component regulatory system; HrpS is a σ^{54}-dependent EBP; HrpL is the master regulator, an ECF sigma factor; RpoN is a sigma 54 factor; YhbH is σ^{54} modulation protein. Thick arrow lines showed gene or operons, oval and circles indicate proteins and arrowheads in thinner lines indicate the directions of information flow. OM, outer membrane; PM, plasma membrane; IM, inner membrane; P, phosphate; RNAP, RNA polymerase; filled triangle, σ^{70} promoter; open triangle, σ^{54} promoter; closed half circle, HrpL promoter.

Sigma factors play important roles in the virulence of *E. amylovora*. We have demonstrated, for the first time, that mutation in *rpoN, hrpL, yhbH,* and *hrpS* gene render *E. amylovora* nonpathogenic. The lack of disease symptoms of all four mutants on both immature pear fruits and apple shoots clearly indicated that sigma factors (RpoN and HrpL), σ^{54}-dependent EBP HrpS, as well as sigma 54 factor modulator (YhbH) are required to cause disease symptoms on host plant. HR assay also illustrated that loss of *rpoN, hrpL, yhbH,* and *hrpS* gene would lead to the loss of HR-eliciting ability of *E. amylovora* as a result of lack of T3SS gene expression.

Gene expression assay further indicated that *rpoN, yhbH* and *hrpS* were all necessary to activate *hrpL* transcription, which in turn activates the expression of other *hrp*-T3SS genes. Mutation in *rpoN, hrpL, yhbH* and *hrpS* gene disrupted the formation of transcription machine, thus paralyzed transcription initiation of *hrpL*. Interestingly, mutation in *rpoN* gene had no effect on *hrpS* expression, and *vice versa.* It seemed that transcription of *rpoN* and *hrpS* was independent from each other. It also appeared that sigma factors and related modulation proteins are required for amylovoran production. *RpoN, yhbH, hrpL* and *hrpS* mutant disrupted the ability of *E. amylovora* to produce normal amount of amylovoran. Gene expression assay verified that these phenotypes were correlated with *amsG* and *rcsA* gene expression. It is possible that there is an interaction between T3SS and amylovoran biosynthesis.

Overexpression of *hrpL* via an arabinose-inducing vector in *rpoN*, *hrpS* and *yhbH* mutant bypassed these genes and rescued *E. amylovora* with HR symptoms on non-host plant tobacco, further indicating that *rpoN*, *hrpS* and *yhbH* genes function at upstream of *hrpL*.

Based on our results, we proposed the following model of regulation of T3SS gene expression in *E. amylovora* (Fig. 2.10). The bacteria may sense environmental stimulus through HrpX/HrpY two-component regulatory system and somehow transmit the signal to HrpS, which needs further study. HrpS is a σ^{54}-dependent EBP, which binds to upstream DNA activator sequences (UAS), located −80 to −150 bp from HrpL transcriptional start site, and forms a hexamer. Meanwhile, RpoN together with core RNAP forms σ^{54}-RNAP complex and binds to −24 (GG) and −12 (TGC) position from HrpL transcription start, and remains transcriptionally silent. With the assistance of integration host factor (IHF), hexametric EBPs contact the σ^{54}-RNAP-promoter complex via the consensus GAFTGA motif and DNA looping. The energy provided by ATP hydrolysis of the EBPs AAA+ domain triggers the formation of σ54-RNAP-promoter complex open, with *hrp*L DNA melting. HrpL is the master switch of *hrp* system and

recognizes a conserved "*hrp* box" at the promoter regions of HrpL-dependent operons or genes and regulates the other *hrp* gene expression. Our results indicate that both RpoN and HrpS are required to active *hrp*L transcription and YhbH is also involved in this process. On the other hand, expression of *rpoN* and *hrpS* is independent from each other.

However, the detailed function of YhbH is still unknown. In *E. coli*, level of σ^{38} protein was observed to be significantly increased when cell growth stops and enters into the stationary phase. Besides, σ^{38} factor assists the function of ribosome modulation factor (RMF), another ribosome-associated protein, in cell viability (Apirakaramwong *et al.*, 1999). It is possible that YhbH aids RpoN in the competition with σ70 since it was found to be involved in 100S ribosome stabilization and preservation (Kato *et al.*, 2010). Studies to determine the molecular mechanism of YhbH function are currently underway.

Chapter 3

Effect of EnvZ/OmpR and GrrS/GrrA Systems on *Erwinia amylovora* Virulence

3.1 Abstract

Two-component signal transduction systems (TCSTs) in *Erwinia amylovora* play a major role in virulence and in regulating amylovoran production, including EnvZ/OmpR and GrrS/GrrA, two widely distributed systems in gamma-proteobacteria. While both systems negatively control amylovoran biosynthesis, deletion mutants of *envZ/ompR* and *grrA/grrS* have opposite swarming motility phenotypes. In order to determine how the two systems interact, two triple mutants, *envZ/ompR/grrA* (*ERA*) and *envZ/ompR/grrS (ERS*) were generated. Our results showed that both triple mutants had slightly increased virulence on apple shoots as compared to that of wild type (WT) as well as mutants deleting a single system. In an *in vitro* amylovoran assay, amylovoran production was significantly increased in the two triple mutants, indicating the two systems synergistically regulate amylovoran production. In consistent with amylovoran production, *amsG* gene expression was expressed significantly higher in the triple mutants *in vitro* than those in WT as well as mutants deleting a single system. In contrast, exopolysaccharide levan was significantly reduced in the triple mutants compared with that of WT and deletion of a single system. In addition, the triple mutants showed reduced swarming motility on swarming plates compared to that of *grrA/grrS* mutants and WT strain, but moved slightly faster than that of *envZ/ompR* mutants, indicating that the two systems antagonistically regulate swarming motility in *E. amylovora*. Furthermore, type III secretion (T3SS) genes were significantly unregulated in the triple mutants as well as deletion of a single system than that of the WT strain. These results indicate that EnvZ/OmpR and GrrS/GrrA systems play major roles in virulence and in regulating virulence gene expression**.**

3.2 Introduction

Erwinia amylovora is the causal agent of fire blight disease on *Rosaceae* plants, especially pears and apples. Fire blight was the first disease attributed to bacterium and *E. amylovora* was the first plant pathogenic bacterium shown to be vectored by insects. Symptoms

of fire blight can be observed on all above ground tissues including blossoms, fruits, shoots, branches and rootstock. After infection, tips of shoots may wilt rapidly to form a typical "shepherd's crook" appearance. Viscous exudates "ooze" containing bacteria and exopolysaccharide (EPS) amylovoran would come out after a while and serve as the source for secondary infection.

E. amylovora is a Gram-negative, rod shaped bacterium with peritrichous flagella. It belongs to the *Enterobacteriaceae* family and is closely related to *Salmonella*, *E. coli* and *Yersinia pestis.* Two major pathogenicity factors in *E. amylovora* have been identified, i.e. EPS amylovoran and type III secretion system (T3SS).

Amylovoran is a complex EPS made up of a large number of repeating units. Amylovoran contributes to *E. amylovora* pathogenesis by protecting the pathogen from plant defense, and binding water and nutrients released from damaged plant cells (Leigh & Coplin, 1992). The ability of individual *E. amylovora* strains to produce amylovoran is positively correlated with the degree of virulence (Ayers *et al.*, 1979). Mutant strains without amylovoran were non-pathogenic (Nimtz *et al.*, 1996). The *ams* (amylovoran biosynthetic) operon, consisting 12 genes (from *ams*A to *ams*L), is responsible for amylovoran biosynthesis. Expression of the *ams* operon is regulated by the Rcs phosphorelay system in *E. amylovora*, which is also essential for pathogenicity (Wang *et al.*, 2009).

In addition to amylovoran, *E. amylovora* also produces another homopolymer EPS, levan, which is composed of β-2, 6-linked fructose molecules. Levan synthesis is mediated by levansucrase (Lsc) (Bereswill & Geider, 1997). In contrast, levan-deficient strains only showed attenuated virulence (Geier & Geider, 1993).

The genome sequence of *E. amylovora* strains revealed three type III secretion systems in *E. amylovora*, including the pathogenicity island1 (PAI-1) containing the hypersensitive response and pathogenicity (*hrp)* T3SS, and two *inv/spa*-like T3SS islands (PAI-2 and PAI-3) (Zhao *et al.*, 2009a). *hrp*-T3SS has been known for its role as a pathogenicity factor that functions to deliver effectors into the eukaryotic host (He *et al.*, 2004). PAI-2 and PAI-3 are similar to the SPI1 T3SS of *Salmonella typhimurium* and the *inv/spa* T3SS of the insect endosymbiont *Sodalis glossidinius*, respectively. However, the role of these T3SSs is still unknown. The pathogenicity of *E. amylovora* requires a functional *hrp*-T3SS in which HrpN and DspA/E were found to play an important role in induction of cell death, activation of defense

pathways, and ROS accumulation. In *E. amylovora*, HrpL is the master switch of the *hrp* system and belongs to a subfamily of bacterial sigma factors. It activates all secretory *hrp* operons, harpin genes and *dsp/avr* genes. In *E. amylovora*, expression of *hrpL* is environmentally regulated and seems partially controlled by HrpS as well as HrpX/Y, a two-component regulatory system.

Two-component signal transduction systems (TCSTs) in *E. amylovora* have been recently found to play a major role in regulating virulence, amylovoran production and swarming motility. EnvZ/OmpR and GrrS/GrrA systems are two widely distributed systems in gamma-proteobacteria (Zhao *et al.*, 2009a). TCSTs normally consists of a membrane-bound histidine kinase (HK) that senses a specific environmental stimulus, and a corresponding response regulator (RR) that mediates the cellular response mostly through differential expression of target genes.

EnvZ/OmpR, as a global and dual regulator, has been found to be involved in regulation of a series of cellar components, virulence and global gene expression. First of all, EnvZ/OmpR is essential for bacterial EPS production in *Salmonella typhi* and *Pseudomonas aeruginosa* (Pickard *et al.*, 1994; Berry *et al.*, 1989). Secondly, OmpR has been suggested to have a positive regulatory effect on flagella master regulator, FlhDC, in *Y. enterocolitica* (Raczkowska *et al.*, 2011). However, in *E. coli* and *Xenorhabdus nematophila*, OmpR was reported as a negative regulator of swarming motility (Kim *et al*., 2003). Furthermore, EnvZ/OmpR has also been reported to regulate genes associated with virulence in several pathogenic bacteria, including *Shigella flexneri, Salmonella typhimurium, Salmonella spp., Yersinia enterocolitica* and *Pseudomonas syringae* (Bernardini *et al.*, 1990; Feng *et al*., 2003; Dorman *et al.*, 1989; Brzostek *et al.*, 2007). Besides, microarray-based comparative transcriptome analysis of *Yersinia pestis* identified a set of 224 genes whose expressions were affected by *omp*R mutation, indicating a global regulatory role in *Y. pestis* (Gao *et al.* 2011).

Similarly, GrrSA system (also called GacSA, BarA/UvrY) has also been reported to regulate an array of phenotypes (Zhao *et al*., 2009b). Two main properties of *gac*S/*gac*A mutants stand out: partial or complete reduced biocontrol ability in a group of plant-beneficial *Pseudomonads* and significantly attenuated virulence in plant- or animal-pathogenic bacteria. GacS/A TCSTs regulate EPS production as well as extracellular enzyme production in a variety species, such as *Pseudomonas fluorescences* CHA0 and *Pectobacterium carotovora subsp.*

carotovora (Cui *et al.*, 2001). As a negative regulator, GacSA was found to down-regulate flagella gene expression in *P. fluorescens, E. coli* and *S. typhimurium* (Teplitski *et al.*, 2003). Mutation in *gac*A gene of *P. fluorescens, P. syringae* B728a and *Dickeya dadantii* showed reduced motile ability (Kato *et al.*, 1989; Kinscherf & Willis, 1999). Moreover, GacS/A controls virulence gene expression in a variety of host-pathogen systems, such as *P. syringae, P. aeruginosa, V. cholera, E. coli, S. enterica,* and *D. dadantii* (Willis *et al.*, 2001; Johnston *et al.*, 1996; Parkins, *et al.*, 2001). In addition, GrrSA in many γ-proteobacteria have been identified to function through the RNA binding protein RsmA and sRNA *rsmB* regulatory system, including *E. coli, P. syringae* and *P. carotovora* (Lapouge *et al.*, 2008). GacSA system positively controls expression of one to five small RNAs (sRNAs), thus upregulating the production of proteins that are otherwise repressed by small RNAs, such as RsmA/CsrA (Cui *et al.*, 2001).

In the last few years, a more complex view of TCSTs came into sight. Given the high level of sequence and structural similarity between different systems, more and more people are trying to link together different TCSTs and define their cross-talks or interference, such as the interaction between CpxA-CpxR and EnvZ/OmpR systems in *E. coli* (Siryaporn & Goulian, 2008). It is possible that cross-talks and interactions between TCSTs enable bacteria to form a regulatory network to properly react towards environmental changes. We have previously reported that both EnvZ/OmpR and GrrSA systems negatively control amylovoran biosynthesis, deletion mutants of *envZ/ompR* and *grrA/grrS* have opposite swarming motility phenotypes (Zhao *et al.*, 2009b). However, we do not know how these two systems interact with each other in *E. amylovora*. The purpose of this study is to determine the interaction of EnvZ/OmpR and GrrS/GrrA on *E. amylovora* amylovoran production, swarming motility and virulence.

3.3 Materials and methods

3.3.1 Bacterial strains and growth condition

The bacterial strains and plasmids used in this study are summarized in Table 3.1. LB medium is used routinely for culture *E. amylovora*. When necessary, the following antibiotics were added to the medium: 50 µg/ml kanamycin (Km), 100 µg/ml ampicillin (Ap) and 20 µg/ml chloramphenical (Cm). Amylovoran production was determined by growing bacteria in MBMA medium (3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $[NH_4]_2SO_4$, 2 ml glycerol, 0.5 g citric acid, 0.03 g MgSO4) amended with 1% sorbitol (Zhao *et al.*, 2009a). A specific *hrp*-inducing minimal medium (HrpMM) (1g $[NH_4]_2SO_4$, 0.246 g $MgCl_2 \cdot 6H_2O$, 0.099 g NaCl, 8.708 g K_2HPO_4, 6.804 g KH_2PO_4) containing 20 mmol galactose was used *in vitro* to mimic conditions of the plant apoplast (Wei *et al.*, 1992).

3.3.2 DNA manipulation and bioinformatics analysis

Standard molecular procedures were followed in the experiment of plasmid DNA purification, PCR amplification of genes, isolation of fragments from agarose gels, cloning, restriction enzyme digestion and T4 DNA ligation (Sambrook & Russell 1989). Protein domain organizations of the HKs and RRs were identified by searching the SMART program http://smart.embl-heidelberg.de/website (Zhao *et al.*, 2009a). Domain limits for proteins were also be derived from the graphical output of the SMART web interface.

Table 3.1: Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characters [a]	Reference or source
E. amylovora **strain**		
Ea1189	Wild type; isolated from apple	Zhao *et al.*, 2009b
Z2946 *ΔflhD*	*flh*D::Km; Km^R-insertional mutant of *flhD* of Ea1189, Km^R	Zhao *et al.*, 2009b
Z0271 *ΔompR*	Km^R-insertional mutant of *ompR* of Ea1189, Km^R	Zhao *et al.*, 2009b
Z0270 *ΔenvZ*	Km^R-insertional mutant of *envZ* of Ea1189, Km^R	Zhao *et al.*, 2009b
Z0270–71 *ΔenvZ/ompR*	Km^R-insertional mutant of *envZ-ompR* operon of Ea1189, Km^R	Zhao *et al.*, 2009b
Z2198 *ΔgrrA*	Km^R-insertional mutant of *grrA* of Ea1189, Km^R	Zhao *et al.*, 2009b
Z3742 *ΔgrrS*	Cm^R-insertional mutant of *grrS* of Ea1189, Km^R	Zhao *et al.*, 2009b
ΔenvZ/ompR/grrA (ERA)	Cm^R-insertional mutant of *envZ/ompR* of Ea1189 *grrA* mutant, Km^R, Cm^R	This study
ΔenvZ/ompR/grrS (ERS)	Cm^R-insertional mutant of *envZ/ompR* of Ea1189 *grrS* mutant, Km^R, Cm^R	This study
ΔgrrS/*grrA*	Cm^R-insertional mutant of *grrS* of Ea1189 *grrA* mutant, Km^R, Cm^R	This study
E. coli **strain**		
DH10B	DH10B F- mcrA Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 ΔlacX74 *recA1 endA1 ara*Δ139 Δ(*ara, leu*)7697 *galU galK* λ – *rpsL* (StrR) *nupG*	Invitrogen, Carlsbad, CA, USA
Plasmids		
pKD46	Ap^R, PBAD gam bet exo pSC101 *oriTS*	Datsenko & Wanner, 2000
pKD13	Km^R, FRT cat FRT PS1 PS2 oriR6K *rgbN*	Datsenko & Wanner, 2000
pKD3	Cm^R, FRT cat FRT PS1 PS2 oriR6K *rgbN*	Datsenko & Wanner, 2000
pGEM® T-easy	Ap^R, PCR cloning vector	Promega, Madison, WI, USA
pWSK29	Ap^R, cloning vector, low copy number	Wang & Kushner, 1991
pFPV25	Ap^R, GFP based promoter trap vector with a promoterless gfpmut3a gene	Valdivia & Falkow, 1997
pAmsG-GFP	A 721 bp *Kpn*I-*Xba*I DNA fragment containing promoter sequence of *amsG* gene in pFPV25	Zhao *et al.*, 2009
pDspE-GFP	A 570bp *Sma*I DNA fragment containing promoter sequence of *dspE* gene in pFPV25	Zhao *et al.*, 2009
pHrpN-GFP	A 608 bp *EcoRI-BamHI* DNA fragment containing promoter sequence of *hrpN* gene in pFPV25	Fan, unpublished

Table 3.1: (Cont.)

Strains or plasmids	Relevant characters [a]	Reference or source
pHrpL-GFP	A 608bp *KpnI-XbaI* DNA fragment containing promoter sequence of *hrpL* gene in pFPV25	Wang *et al.*, 2010
pYsaE1-GFP	A 774 bp *EcoRI-BamHI* DNA fragment containing promoter sequence of *ysaE1* gene in pFPV25	Nakka *et al.*, 2010
pYsaE2-GFP	A 774 bp *EcoRI-BamHI* DNA fragment containing promoter sequence of *ysaE2* gene in pFPV25	Nakka *et al.*, 2010
pPrgH2-GFP	A 618 bp *EcoRI-BamHI* DNA fragment containing promoter sequence of *prgH1* gene in pFPV25	Nakka *et al.*, 2010
pPrgH1-GFP	A 618 bp *EcoRI-BamH I*DNA fragment containing promoter sequence of *prgH2* gene in pFPV25	Nakka *et al.*, 2010

a. Km^R =Kanamycin resistance, Ap^R =ampicillin resistance, Cm^R= chloramphenical resistance;

3.3.3 Construction of TCST mutants in *E. amylovora* by Lambda-Red recombinase

Lambda phage recombinases were used to generate *E. amylovora* stable mutants as previously described (Zhao *et al.*, 2009a). Briefly, *E. amylovora* Δ*envZ/ompR* or Δ*grrA/S* mutants were transformed with plasmid pKD46 expressing recombinases red α, β, and γ. The transformant Δ*envZ/ompR* or Δ*grrA/S* (pKD46) were grown overnight at 28°C, reinoculated in LB broth containing 0.1% arabinose, and grown to exponential phase OD_{600}=0.8. Cells were collected, made electro-component, and stored at -80°C. Recombination fragments consisting of a kanamycin (*kan*) or chloramphenical (*cat*) gene with its own promoter, flanked by a 50-nucleotide (nt) homology arm, was generated by PCR using plasmid pKD13 or pKD3 as a template. The primers that used for generating mutants are listed in Table 3.2. Primers and internal primer pair Km1 and Km2 of the *kan* gene, Cm1 and Cm2 of the *cat* gene were used to confirm mutants by PCR. For the resulting mutants, the majority of the coding region of each gene was replaced by the *kan* or *cat* gene, except for the first and last 50 nt. The resulting mutants were designated and listed in Table 3.1.

Table 3.2: Primers used in this study

Primer	Sequences (5'—3')	Reference or source
B0270R	TTATGCCACCGGGCCGGCCGGCAAGCTGGCTGGCGGCAGCG GCAGATAAG ATTCCGGGGATCCGTCGACC	Zhao *et al.*, 2009b
B0271F	ATGAAAGATAAGCTGCTGTTTAATATGCTTTGTAACAATTTCG GCTACAA GCGATTGTGTAGGCTGGAGCT	Zhao *et al.*, 2009b
B2198F	TTGATTAGCGTTTTTCTTGTTGATGACCATGAGCTGGTGCGCG CAGGTAT GCGATTGTGTAGGCTGGAGCT	Zhao *et al.,* 2009b
B2198R	TTACTCACTACTGATTAATGACTCCGCACTGAACAGACCATGT CGAATGG ATTCCGGGGATCCGTCGACC	Zhao *et al.*, 2009b
B3742F	ATGACCAAATACAGCCTGCGGGCACGCATGATGATTTTGATT CTGGCACCGCGATTGTGTAGGCTGGAGCT	Zhao *et al.,* 2009b
B3742R	TTACAGCTTCAGCCGCTCTTTGGCCAGTCGTGCCACGTTATGC ATTTCGTCCATGGTCCATATGAATATCCTCC	Zhao *et al.,* 2009b
Z270 R	ATCTGGCCCGGCGTTTTATCA	Zhao *et al.,* 2009b
Z271 F	CGTGGTTATGCCGCTATTGTGTTG	Zhao *et al.,* 2009b
Z2198 F	TTACCCGTTATTTGCAGTTGTTCCG	Zhao *et al.,* 2009b
Z2198 R	TGGGTTACCGTCACGTCTATCTGC	Zhao *et al.,* 2009b
Z3742 F	CGTTATTGTCTGGCGGGTCGTCAC	Zhao *et al.,* 2009b
Z3742 R	CGTTACAGGAAGCAGCGGAGAATG	Zhao *et al.,* 2009b
Km1	CAGTCATAGCCGAATAGCCT	Zhao *et al.,* 2009a
Km2	CGGTGCCCTGAATGAACTGC	Zhao *et al.,* 2009a
Cm1	TTATACGCAAGGCGACAAGG	Zhao *et al.,* 2009a
Cm2	GATCTTCCGTCACAGGTAGG	Zhao *et al.,* 2009a
16S1	CCTCCAAGTCGACATCGTTT	Wang et al., 2009
16S2	TGTAGCGGTGAAATGCGTAG	Wang *et al.*, 2011b
amsG-rt1	CAAAGAGGTGCTGGAAGAGG	Wang *et al.,* 2011b
amsG-rt2	GTTCCATAGTTGCGGCAGTT	Wang *et al.,* 2011b
amsD-rt1	GATGCGTCTGTTCAAGCTGT	Wang *et al.,* 2011b
amsD-rt2	TCGCAACAAATCAGTCTGGA	Wang *et al.,* 2011b
rcsA-rt1	TTAAACCTGTCTGTGCGTCA	Wang *et al.,* 2011b
rcsA-rt2	AGAAACCGTTTTGGCTTTGA	Wang *et al.,* 2011b
dspE-rt1	TCCAGCGAGGGCATAATACT	Wang *et al.,* 2011b
dspE-rt2	ACAACCGTACCCTGCAAAAC	Wang *et al.,* 2011b
hrpL-rt1	TTAAGGCAATGCCAAACACC	This study
hrpL-rt2	GACGCGTGCATCATTTTATT	This study
hrpN-rt1	GCTTTTGCCCATGATTTGTC	Wang *et al.,* 2011b
hrpN-rt2	CAACCCGTTCTTTCGTCAAT	Wang *et al.,* 2011b
glgB-rt1	GGGTTCAATTCTCGACCGTA	Wang *et al.,* 2011b
glgB-rt2	GGTGTCGTGGTTCCACTCTT	Wang *et al.,* 2011b

3.3.4 Virulence assays on apple plants and immature pear fruit

In brief, *E. amylovora* WT and mutant strains were grown overnight in LB broth, harvested by centrifugation, and resuspended in ½*sterile phosphate buffered-saline (PBS) with bacterial cells adjusted to OD_{600} = 0.001 in ½*PBS. Immature fruits of pear (*Pyruscommunis* L. cv Bartlett) were surface-sterilized with 10% bleach, dried in a flow hood and pricked with a sterile needle followed by the standard procedure (Zhao *et al.*, 2005). Two µl of cell suspensions was inoculated on the wounded tissue and incubated pears in a humidified chamber at 26°C. Symptoms were recorded at 4 and 8 days post-inoculation. For each strain tested, fruits were assayed in triplicate, and each experiment was performed at least three times.

Apple shoot virulence assays were performed on young annual shoots of "gala" apple, 22 to 25 cm in length. After pricking the tip with a sterilized needle, five µl of pathogen suspension with an initial OD_{600}=0.1 was pipetted onto the wounded tissue. For each bacterial strain, seven shoots were inoculated. Plants were kept in a greenhouse at 25°C and a 16 hours light photoperiod, and periodically evaluated for disease development for up to 7 days following inoculation by measuring length of the necrotic tissue. The experiment was performed at least three times.

3.3.5 CPC assay for determining amylovoran concentration

The amylovoran concentration in supernatants of bacterial cultures was quantitatively determined by a turbidity assay with cetylpyrimidinium chloride (CPC) as described (Hildebrand *et al.*, 2006). Briefly, *E. amylovora* WT and mutant strains were grown overnight in LB broth and washed with ½*PBS three times. After the final wash, the bacteria pellet was resuspended in 200 µl PBS and inoculated into 5ml MBMA+1% sorbitol medium with an initial OD_{600}=0.2. One ml of bacterial cells was pelleted 24 hours after inoculation at 28°C with shaking. Following centrifugation, 50 µl CPC at 50 mg /ml was added to one ml supernatant, incubated 10 min at room temperature, and determined amylovoran concentration by measuring OD_{600} turbidity. The final concentration of amylovoran production was normalized for a cell density of 1.0. For each strain tested, the experiment was repeated at least three times.

3.3.6 Levan production

Levansucrase activity was carried out as previously described (Bellemann *et al.*, 1994). For *E. amylovora* WT and mutants, bacteria were grown overnight in LB broth, harvested by centrifugation. One ml of cell-free supernatant was added to 1 ml of LS buffer (50 mM Na_3PO_4, 2 M sucrose, and 0.05% NaN_3). After 24 h of incubation at 37°C, absorbance was determined at OD_{590}. Each experiment included four biological replicates per strain, and the experiment was performed three times.

3.3.7 Swarming motility assay

For *E. amylovora* WT and mutant strains, bacteria were grown overnight in LB broth, harvested by centrifugation, washed with PBS once, and resuspended in 200 µl PBS. Then, bacterial suspensions were diluted 100×water, and five µl of the diluted bacterial suspension was plated onto the center of swarming agar plates (10 g tryptone, 5 g NaCl, 3 g agar per l Liter) as previously described (Zhao *et al.*, 2009b； Skerker *et al.*, 2005). Swarming diameters were determined following incubation at 28°C for up to three days. The experiment was repeated at least three times.

3.3.8 GFP reporter gene assay by flow cytometry

The BD FAC SCanto flow cytometer was used to monitor GFP intensity of WT and mutant strains containing the corresponding promoter-GFP construct (Zhao *et al*., 2009b). For *in vitro amsG* gene expression, WT and mutants containing the *amsG* promoter-GFP fusion plasmid were grown in LB overnight, harvested, and resuspended in ½*PBS. Bacterial suspensions were reinoculated in MBMA broth with 1% sorbitol and grown at 28°C with shaking for up to two days. Bacterial cultures were then harvested by centrifugation, washed once with ½*PBS, and resuspended in PBS for flow cytometry assay. For *in vitro dspE, hrpL,* and *hrpN* gene expression, WT and mutants containing the *dspE*, *hrpL* and *hrpN* promoter-GFP fusion plasmid were grown in LB overnight, harvested, and resuspended and washed twice with *hrp*-inducing minimal medium and then resuspended in ½*PBS, respectively. Bacterial suspensions then were reinoculated in *hrp*-inducing minimal medium and grown at 18°C with shaking for 18 hours for flow cytometry assay. For *in vitro ysaE1, ysaE2, prgH1* and *prgH2* gene

expression, WT and mutants containing the corresponding gene promoter-GFP fusion plasmid were grown in LB overnight, harvested and resuspended in ½*PBS, respectively. Bacterial suspensions were reinoculated in LB broth and grown at 28°C with shaking for 24 hours. Flow cytometry was performed on a BD LSRII 10 parameter multi-laser analyzer (BD Bioscience, San Jose, CA). For both cases, data were collected for a total of 100,000 events and statistically analyzed by gating using flow cytometry software FCS Express V3 (De Novo Software, LA, CA). A geometric mean was calculated for each sample. Each treatment was performed in triplicates and each experiment was repeated three times.

3.3.9 RNA isolation

For *in vitro* assay, bacterial strains were grown overnight in MBMA+1% sorbitol medium at 28°C for 24 hours or *hrp*-inducing minimal medium at 18°C for 18 hours. For *in vivo* assay, bacterial strains were collected from inoculated pear fruits 18 hours after inoculation as described above. For both cases, four mL of RNA Protect Reagent (Qiagen) were added to two ml bacterial cultures (at OD_{600} of about 0.5–0.8) to stabilize RNA, harvested the cells by centrifugation for 10 min at 4000 g and extracted RNA using Qiagen Bacterial RNA Mini Kit. Dnase (Qiagen, Hilden, Germany) was used to eliminate residue genomic DNA by an on-column digestion method.

3.3.10 Quantitative real-time PCR (qRT-PCR)

To validate the CPC turbidity data and disease symptoms, qRT-PCR was performed as previously described (Wang *et al.*, 2011) to compare the relative expression of target genes of *E. amylovora* Δ*envZ/ompR* and Δ*grrS/A* mutants with WT strain. For each sample, synthesis of cDNA was performed with one µg of total RNA and SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Primers were designed using Primer3 software with high specificity and were listed in Table 3.2. qRT-PCR was conducted in the ABI 7300 System (Applied Biosystems) using Fast SYBR Green PCR master mix (Applied Biosystems). All reactions were run on 96-well optical reaction plates. One µl of cDNA (2 ng/ reaction) or water (no-template control) was used as template for qPCR reactions with Power SYBR Green PCR Master Mix (Applied Biosystems) with a final primer concentration of 500nmol. Primers in Table 3.2 were used to detect the expression of *E. amylovora amsG, amsD, rcsA, glgB, dspE,*

hrpL, and *hrpN* gene, respectively. Technical replicate experiments were performed for each biological triplicate sample. The thermal cycling conditions included a step of 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The relative quantification (ΔΔCt) method was used to determine expression level of selected genes and 16S rRNA (*rrsA*) gene was used as an endogenous control to normalize our samples ($\Delta Ct = Ct_{target} - Ct_{rssA}$). A relative quantification (RQ) value was calculated as $2^{-(\Delta\Delta Ct = \Delta Ctttarget - \Delta Ctreference)}$ for each gene with the control group as a reference. A p-value was computed using a moderated t-test to measure the significance associated with each RQ value. Variations were considered statistically significant when the p-value was <0.05. RQ values for *envZ/ompR* and *grrS/A* single, double and triple mutants were then normalized to those of WT (Wang *et al.*, 2011).

3.3.11 Statistical analysis

One-way ANOVA and Student-Newman-Keuls test were used to determine differences in virulence progress, amylovoran production, gene expression and swarming motility data means within $a = 0.05$, analyzed by SAS 9.2 program.

3.4 Results

3.4.1 Generation of *envZ/ompR* and *grrS/grrA* mutants

The gene organization of EnvZ/OmpR and GrrSA systems are conserved among enterobacteria, in which the *ompR-envZ* operon is co-transcribed, whereas *grrS* and *grrA* are separate from each other in the genome. EnvZ is a sensor kinase which contains a conserved histidine kinase domain (HisKA). OmpR is the corresponding response regulator and contains a conserved receiver domain (REC). However, GrrS is quite different in that GrrS is an unorthodox hybrid sensor kinase that contains a conserved histidine kinase domain (HisKA), a receiver domain and an HPt domain. Once sense the environmental stimulus, GrrS transfers a phosphoryl group from its HisKA domain to its REC domain and then to the HPt domain, which further transfer to the aspartate group of GrrA (Fig 3.1 A) (Workentine *et al.*, 2006).

In order to study the function of EnvZ/OmpR and GrrS/GrrA systems in the virulence of *E. amylovora* as well as interaction between the two systems, we generated three non-polar insertional mutants within *envZ/ompR* and *grr*S/*grr*A genes (Zhao *et al.*, 2006), including one double mutant (*grr*S/*grr*A), and two triple mutants, i.e. *envZ/ompR/grr*S (*ERS*) and *envZ/ompR/grr*A (*ERA*) (Fig 3.1 B).

3.4.2 EnvZ/OmpR and GrrS/GrrA systems synergistically suppress amylovoran production in vitro

Previously, we have reported that EnvZ/OmpR and GrrS/GrrA systems negatively regulated amylovoran production *in vitro* (Zhao *et al*., 2009b). Amylovoran production for the two triple mutants as well as single or double mutants was measured *in vitro*. At 24 and 48 hours post inoculation, all eight mutants showed a dramatic increase in amylovoran production compared to that of the WT (about 0.1) (Fig 3.2 A). The *env*Z, *omp*R, *env*Z/*omp*R, *grr*A, *grr*S and *grr*S/*grr*A mutants produced approximately 16-fold and 8-fold more amylovoran compared with that of the WT at 24 and 48 h, respectively. The two triple mutants produced even more amylovoran compared to those of the single and double mutants. These results indicated that both EnvZ/OmpR and GrrS/GrrA acted as negative regulators of amylovoran production and their effect on amylovoran production *in vitro* were synergistic.

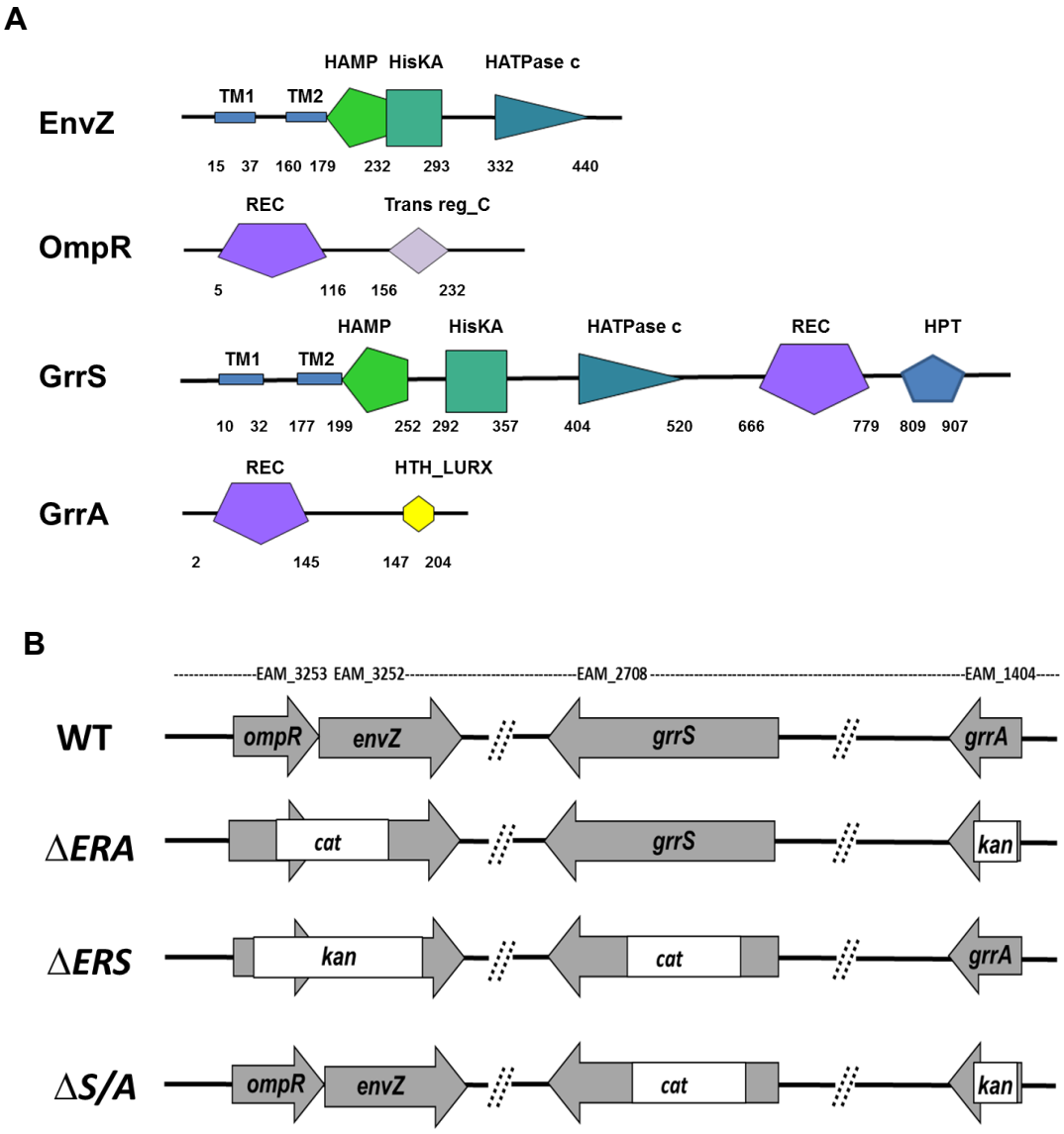


Figure 3.1 Schematic map of genes and proteins of EnvZ/OmpR and GrrS/GrrA systems in *Erwinia amylovora* and generation of insertional *ERA, ERS* and *grrS/grrA* deletion mutants. A) Domain organization of EnvZ, OmpR, GrrS and GrrA proteins. Domain limits for proteins are derived from the SMART program. Not drawn to scale. HisKA, Histidine Kinase A domain; REC, Receiver domain; HATPase_c, Histidine kinase-like ATPases; HPT: Histidine Phosphotransfer domain; HTH_LUXR domain, helix_turn_helix, Lux regulon. B) Organizations of *envZ, ompR, grrS* and *grrA* genes and generation of insertional *ERA*, *ERS* and *grrS/grrA* deletion mutants. Arrowheads show orientations. *kan*, kanamycin resistant gene; *cat*, chloramphenicol resistant gene.

We also determined levan production in those eight mutants. *E. amylovora* wild type produced levan at 0.35 after normalization. Levan production for *envZ*, *ompR* and *envZ/ompR*, *grrA*, *grrS* and *grrS/grrA* mutants was 0.21, 0.20, 0.16, 0.02, 0.02, and 0.02, respectively (Fig 3.2 B). The two triple mutants produced similar amount of levan as compared to *grrS/A* mutants, indicating that both systems were required for levan production and the suppression effect of GrrSA system on levan production was dominant.

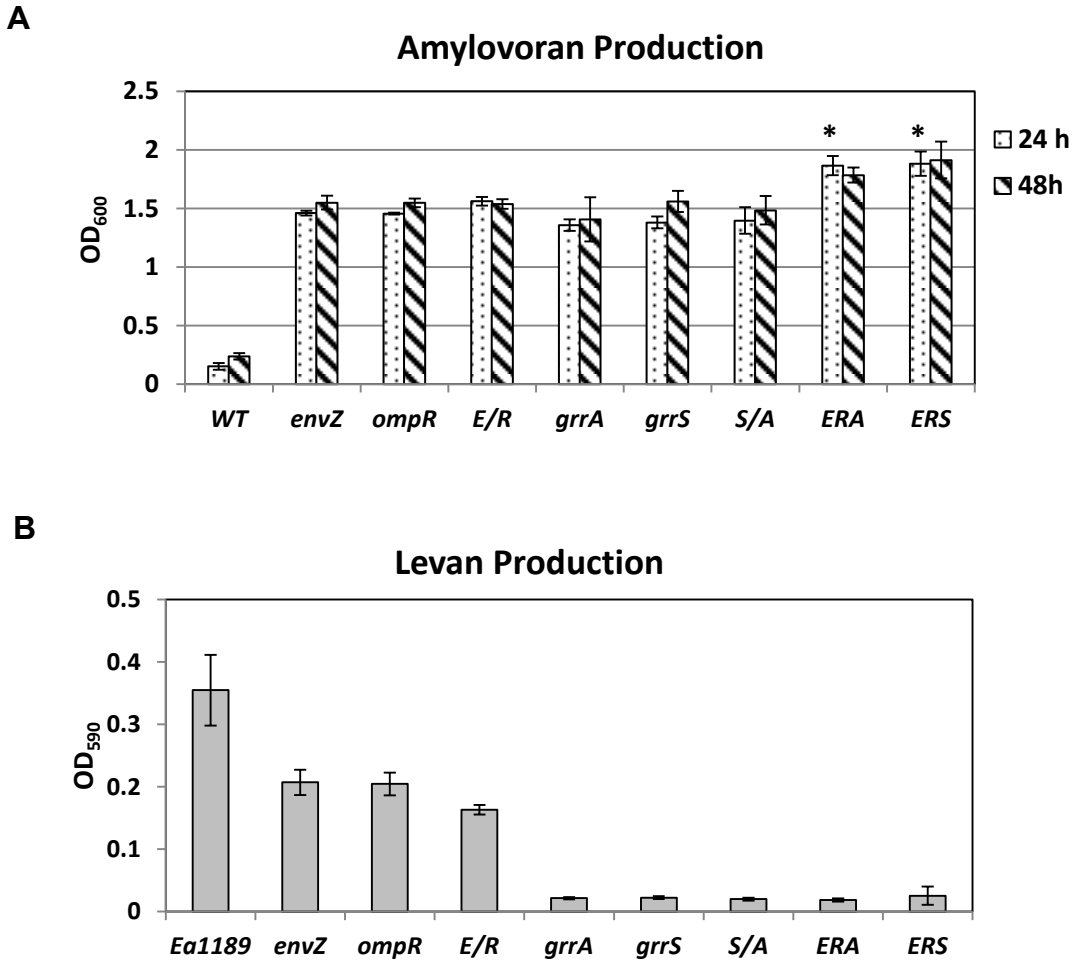


Figure 3.2 Exopolysaccharide amylovoran (A) and levan (B) production of *Erwinia amylovora* WT and mutant strains. *, statistically different from single and double mutants, α=0.05

3.4.3 EnvZ/OmpR and GrrS/GrrA systems antagonistically regulate swarming motility

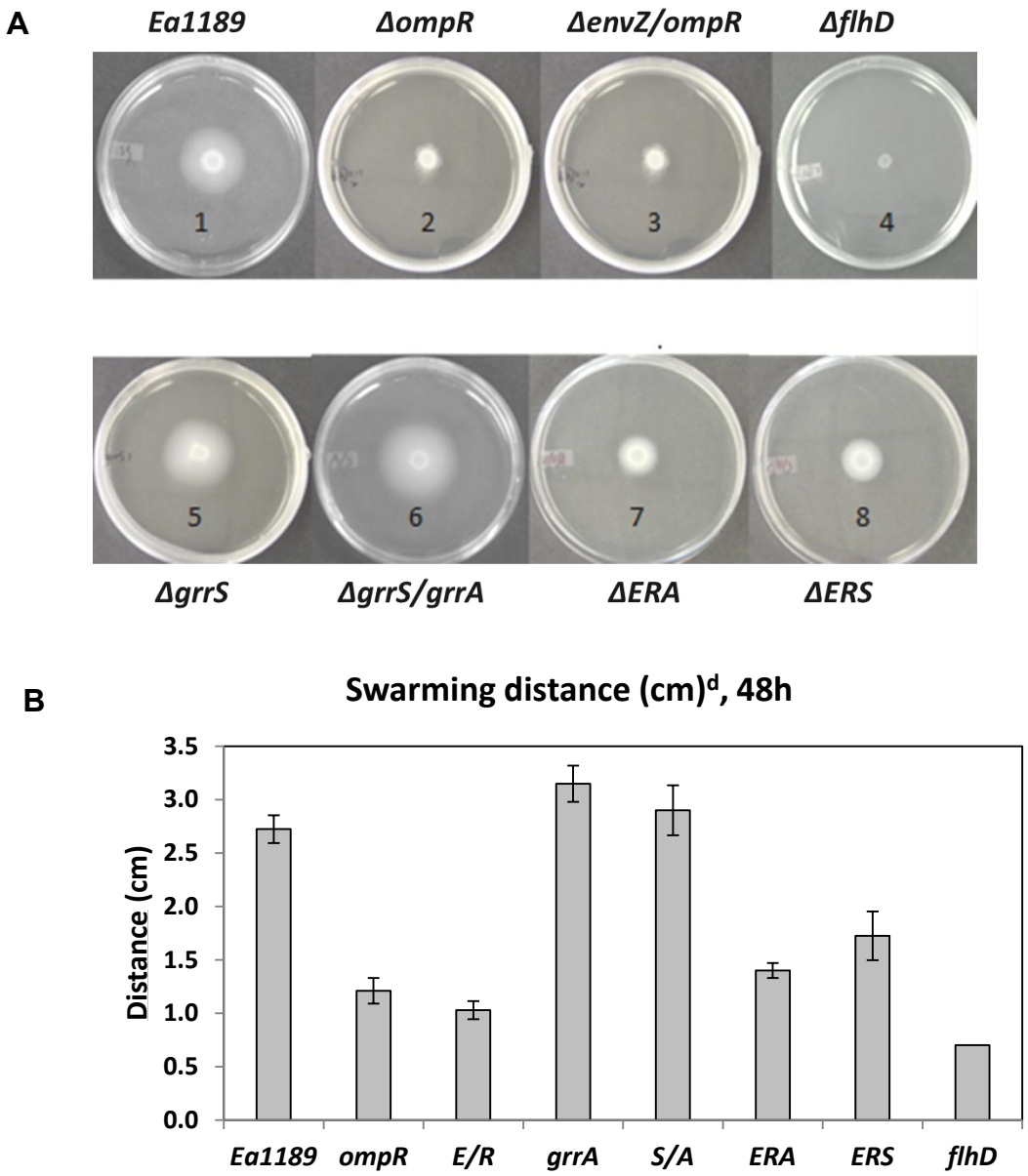


Figure 3.3 Swarming motility assay of *Erwinia amylovora* WT and mutant strains. A) Comparison of motility on swarming plates for WT and mutant strains. Bacterial strains were spotted on swarming plate (0.3% agar) and incubated at 28°C. Photos were taken at two days post inoculation. 1 to 8: WT strain, *envZ*, *envZ/ompR*, *flhD*, *grrS*, *grrS/grrA*, *ERA* and *ERS* mutants, respectively. Flagella *flhD* mutant (4) was used as negative control. B) Comparison of the swarming distance of WT and mutant strains. The diameters of the swarming circle (cm) were measured 48h post incubation.

In our previous study, we have reported that EnvZ/OmpR system acts as a positive regulator and GrrS/A system as a negative regulator of swarming motility (Zhao *et al.*, 2009b). Motility of the two triple mutants was evaluated using a swarming plate assay. As expected, *grrS/grrA* double mutant strains showed precocious swarming and moved faster than the WT strain as *grr*A single mutants, with a diameter of 3.0 cm within 48 hours compared to 2.7 cm for the WT strain, from an original spot of 0.6 cm in diameter (Fig 3.3A). Two triple mutants showed intermediate circular and swarming phenotype (Fig 3.3 B), i.e. the swarming distances were 1.5 and 1.8 cm at 48 hours. The data clearly indicate that the regulatory role of EnvZ/OmpR and GrrS/A on *E. amylovora* swarming motility and further suggested that their effect were antagonistic.

3.4.4 Mutations in *envZ/ompR* and *grrS/grrA* render Erwinia amylovora slightly more pathogenic

To determine the role of EnvZ/OmpR and GrrS/GrrA systems in *E. amylovora* virulence, we conducted virulence assays for *envZ/ompR*, *grrS/grrA*, *ERS* and *ERA* mutants on both immature pear fruits and apple shoots (Zhao *et al*., 2005; Zhao *et al.*, 2006). At 2 days following inoculation, *E. amylovora* WT strain Ea1189 produced water soaking symptoms on pears with visible bacterial ooze. Four days after inoculation, Ea1189-inoculated immature pears showed necrotic lesions and bacterial ooze formation. After 8 days, the necrosis turned black with more ooze production at the inoculation site. Similar disease progresses were observed on immature pear fruits inoculated with *envZ/ompR* and *grrS/grrA* double mutants as WT strain (Fig 3.2). Meanwhile, similar disease symptom and ooze formation was observed on immature pear fruits inoculated with the two triple mutants.

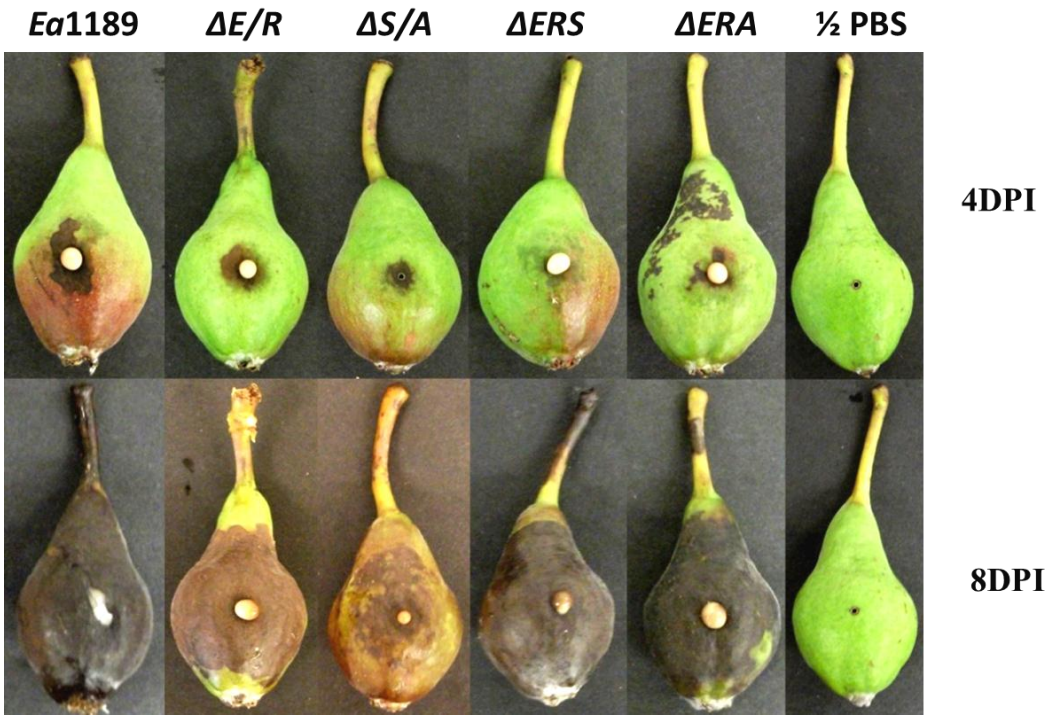
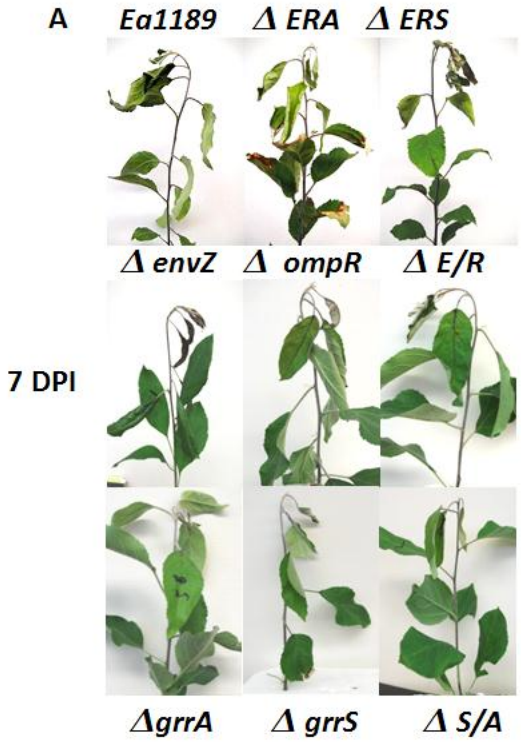


Figure 3.4 Pathogenicity tests of *Erwinia amylovora* WT and mutant strains on immature pears. Symptoms caused by WT, *env*Z/*omp*R, *grr*A/*grr*S, *ERS* and *ERA* on immature pears after 4 and 8 days. DPI, days post-inoculation. ½*PBS was used as negative control.

Virulence of *envZ/ompR* and *grrS/grrA* mutants was also tested on "gala" apple shoots (Fig 3.4).WT strain caused visible necrosis around the inoculated site 2 DPI and quickly spread throughout the leaf and into petioles and finally, into the shoot, and covered 17.0 cm at 7 DPI (Fig 3.4). *envZ* and *envZ/ompR* mutants showed similar disease progresses as WT. Slightly severer disease symptom was observed for *grr*S and *grr*A/*grr*S double mutants. Furthermore, *ERS* and *ERA* triple mutants showed slightly increased virulence on "gala" shoots and the mean lengths of diseased tissues were 21.44 and 20.41 cm at 7 DPI (Fig 3.4). These results indicate that both EnvZ/OmpR and GrrA/S systems contributed to bacterial virulence.

A



B

Length of necrosis lesion (cm)

	4d	5d	6d	7d
Ea1189	6.63 ± 1.63	9.13 ± 2.61	13.7 ± 3.29	17.22 ± 3.45
ΔenvZ	6.17 ± 1.93	9.11 ± 2.03	13.13 ± 2.41	18.66 ± 2.31
ΔE/R	8 .00± 2.55	9.9 ±1.63	12.33 ± 5.92	18.05 ± 4.24
ΔgrrA	9.13 ± 2.30	11.58 ± 3.32	16.59 ± 2.35	19.1 ± 3.02
ΔS/A	9.54 ± 2.61	12.57 ± 2.00	15.6 ± 3.72	18.86 ± 3.52
ΔERA	10.25 ± 3.53	13.33 ±1.95	19.67 ±2.62	21.44 ± 2.21
ΔERS	10.73 ± 2.92	13.53 ± 2.48	18.32 ± 2.38	20.41 ± 2.49

Figure 3.5 Virulence assay for *Erwinia amylovora* WT and mutant strains. A) Symptoms on 'gala' apple shoots. Pictures were taken 7 days post inoculation. B) Progression of necrosis on inoculated 'gala' shoots. Apple shoots were inoculated with 5µl of bacterial suspension (OD_{600}=0.1). Disease severity was measured and recorded at 4, 5, 6, 7 days post inoculation. For each strain, the necrosis length was the mean of seven replicates.

A

	*ams*G	*dsp*E	*hrp*L	*hrp*N
***Ea*1189**	3.25 ± 0.42	1.99 ± 0.16	3.89 ± 0.33	2.60 ± 0.17
ΔenvZ	70.18 ± 2.73	20.94 ± 1.04	71.86 ± 0.93	14.87 ± 1.56
ΔompR	79.63 ± 17.02	25.28 ± 0.47	74.29 ± 1.18	16 ± 0.89
ΔE/R	67.64 ± 3.22	16.82 ± 0.81	51.03 ± 17.39	12.06 ± 0.64
ΔgrrA	116.01 ± 15.81	99.81 ± 1.31	126.48 ± 0.97	32.33 ± 1.57
ΔgrrS	74.89 ± 4.56	108.86 ± 6.14	154.26 ± 5.73	30.86 ± 0.94
ΔS/A	81.9 ± 10.52	92.10 ± 1.41	158.68 ± 9.34	30.24 ± 0.41
ΔERA	420.23 ± 53.23	192.20 ± 4.49	327.99 ± 8.48	84.37 ± 3.79
ΔERS	432.01 ± 35.11	172.99 ± 1.13	320.18 ± 14.44	61.73 ± 1.25

B

	*ysaE*1	*ysaE*2	*prgH*1	*prgH*2
***Ea*1189**	2.35 ± 0.28	5.38 ± 0.57	2.67 ± 0.36	4.39 ± 0.73
ΔompR	4.63 ± 0.20	29.92 ± 4.94	4.85 ± 0.07	14.95 ± 0.90
ΔE/R	5.81 ± 0.43	42.59 ± 5.99	3.61 ± 0.03	71.22 ± 0.97
ΔgrrA	8.72 ± 1.06	278.75 ± 11.42	10.67 ± 0.45	55.72 ± 1.72
ΔS/A	10.56 ± 1.30	264.37 ± 24.01	12.33 ± 0.41	50.64 ± 1.57
ΔERA	12.09 ± 0.60	255.48 ± 11.09	16.70 ± 1.85	56.52 ± 2.92
ΔERS	12.54 ±1.66	323.87 ± 28.53	17.05 ± 0.40	91.79 ± 4.75

Table 3.3: Promoter activities of *ams*G, *dsp*E, *hrp*L, *hrp*N, *ysaE*1, *ysa*E2, *prgH*1 and *prg*H2 genes in *Erwinia amylovora* WT and mutant strains. GFP intensity in WT and mutant strains containing *ams*G, *dsp*E, *hrp*L, *hrp*N, *ysaE*1, *ysa*E2, *prg*H1 and *prg*H2promoter-GFP fusion plasmids were measured by flow cytometry.

3.4.5 EnvZ/OmpR and GrrS/GrrA systems synergistically regulate expression of amylovoran biosynthetic and T3SS genes *in vitro*

To correlate amylovoran production with amylovoran biosynthesis gene expression, promoter activity of *amsG* gene was measured using GFP as a reporter in WT and eight mutants using flow cytometry (Zhao *et al.*, 2009b). The *ams*G gene was expressed at a basal level in WT strain, with a GFP intensity value of 3.25 (geometric mean), compared to a geometric mean value of 1.5 for the vector control. The intensity of the *amsG* promoter was 70.18, 79.63, 67.64, 116.01, 74.89 and 81.9 for *env*Z, *omp*R, *env*Z/*omp*R, *grr*S, *grrA* and *grr*S/*grr*A mutants, respectively. A significant increase in *amsG* promoter activity of two triple mutants (*ERS*, *ERA*) was observed**,** with a geometric mean value of 432.01 and 420.23, respectively (Table 3.3). These results indicated that there is a synergistic effect of EnvZ/OmpR and GrrA/S systems on amylovoran biosynthetic gene expression in *E. amylovora*.

Next, we determined the promoter activities for three T3SS genes in WT and mutant strains. T3SS genes were expressed at a basal level in WT strain, with a GFP intensity value of 1.99, 3.89 and 2.60 (geometric mean) for *dspE, hrpL* and *hrpN,* respectively (Table 3.3 A). The geometric mean values of the GFP intensity for *dspE, hrpL* and *hrpN* promoters were all increased in *env*Z, *omp*R, *env*Z/*omp*R, *grr*S, *grrA* and *grr*S/*grr*A mutants. For the two triple mutants, geometric mean values of the GFP intensity for *dsp*E, *hrp*L and *hrp*N promoters reached highest at192.20, 327.99, 84.37 in *ERS* mutant, and 172.99, 320.18 and 61.73 in *ERA* mutant, respectively (Table 3.3A). These data indicated that EnvZ/OmpR and GrrA/S systems negatively regulate T3SS gene expression *in vitro* in a synergistic way.

The promoter activities of two other PAIs (PAI-2 and PAI-3) T3SS genes, including *ysaE*1, *ysa*E2, *prg*H1 and *prg*H2 were also measured by GFP-promoter fusion reporter in LB medium. These genes were expressed at a basal level in WT strain, with a GFP intensity value of 2.35, 5.38, 2.67 and 4.39 (geometric mean) for *ysaE*1, *ysa*E2, *prg*H1 and *prg*H2, respectively (Table 3.3 B). Interestingly, all six (2 single, 2 double and 2 triple) mutants showed increased GFP intensity value for all four genes, especially in the triple mutants. These results suggest that PAI-2 and PAI-3 genes were all negatively regulated by EnvZ/OmpR and GrrSA systems *in vitro* in similar way as other *hrp*-T3SS genes.

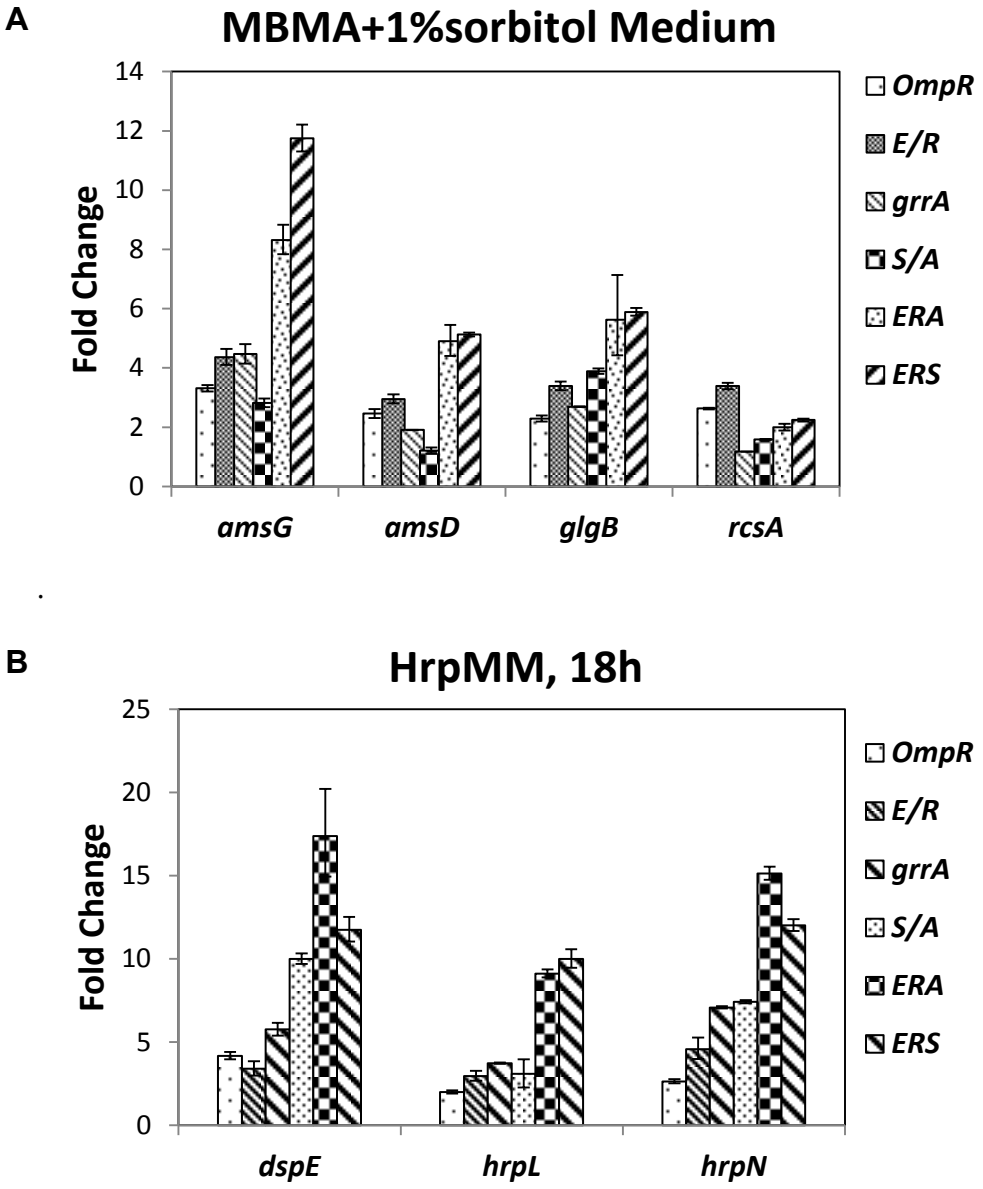


Figure 3.6 Gene expression of selected genes determined by qRT-PCR *in vitro*. The relative fold change of each gene was derived from the comparison of each mutant versus wild type in MBMA +1% sorbitol medium (A), and Hrp inducing minimal medium (B). 16S rRNA gene was used as endogenous controls.

qRT-PCR was also performed to confirm the *in vitro* promoter activity results. Total mRNA of WT and mutant strains extracted from MBMA+1% sorbitol medium was used to test the expression of amylovoran biosynthetic genes (*amsG* and *amsD*), amylovoran regulatory gene

(*rcsA*) and glycogen branching enzyme gene (*glgB*). As expected, all genes were upregulated in six mutants more than two folds. It also revealed that *amsG* was up-regulated by 5.3- and 11.7-fold in *ERA* and *ERS* mutants, respectively. Similarly, *amsD* was up-regulated 4.9- and 5.1- fold, respectively in the two triple mutants. Expression of *glgB* gene showed similar increase as *amsD* gene with more than two folds in the six mutants (Fig 3.6A). These results suggested that EnvZ/OmpR and GrrSA synergistically affect amylovoran gene expression.

Consistently, T3SS genes (*dspE, hrpL* and *hrpN*) showed up-regulated expression in *ompR, envZ/ompR, grrA, grrS/grrA, ERA* and *ERS* mutants as compared with WT. Expression of *dspE* was up-regulated about 4.2-, 3.4-, 5.8-, 10.0-, 17.4- and 11.7-fold higher in the six mutants than wild type, respectively. Similarly, expression of *hrpL* was up-regulated by 2.0-, 3.0-, 3.7-, 3.1-, 9.1- and 10- fold in *ompR, envZ/ompR, grrA, grrS/grrA, ERA* and *ERS* mutants, respectively. Furthermore, expression of *hrpN* was significantly higher in the *ERA* and *ERS* mutants, up15.1- and 12.0-fold, as compared to 2.6- to 7.4-fold increases in *ompR*, *envZ/ompR*, *grrA* and *grrS/grrA* mutants (Fig 3.6 B). These data verified the synergistic effect of EnvZ/OmpR and GrrSA on T3SS gene expression.

3.4.6 EnvZ/OmpR and GrrS/GrrA systems synergistically regulate expression of T3SS genes *in vivo*

As shown in Fig 3.7, all genes, except for the *amsG* and *hrpL,* was slightly upregulated in the six mutants. For example, expression of *amsD* was 1.8-, 1.9-, 2.1-, 1.7-, 2.4- and 3.0- fold higher in the *ompR, envZ/ompR, grrA, grrS/grrA, ERA* and *ERS* mutants as compared to the WT, respectively, while *amsG* expressed only slightly higher than the WT strain. Meanwhile, *glgB* gene expressed more than two folds higher in the six mutants than in WT. Similarly, expression of *dspE* and *hrpN* was up-regulated in *ompR, envZ/ompR, grrA, grrS/grrA, ERA* and *ERS* mutants. However, expression *hrpL* didn't appear to be significant different between the six mutants and WT at 18 h (Fig 3.7).

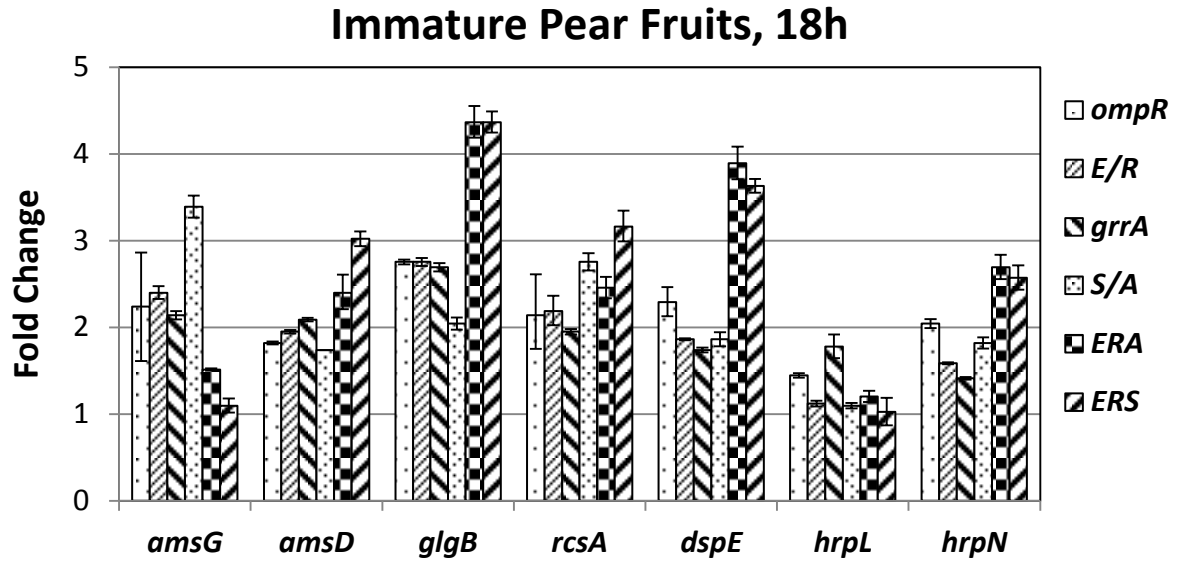


Figure 3.7 Gene expression of selected genes determined by qRT-PCR *in vivo*. The relative fold change of each gene was derived from the comparison of each mutant versus wild type on immature pear fruit. 16S rRNA gene was used as endogenous controls.

3.5 Discussion

There are two major pathogenicity factors in *E. amylovora*, the EPS amylovoran and *hrp*-T3SS. The bacterium utilizes amylovoran to protect from plant defense, and bind to water and nutrients. Meanwhile, *hrp*-T3SS functions to deliver effector proteins into host cell or apoplast to cause disease. Two-component signal transduction systems play an important role in regulating *E. amylovora* virulence, amylovoran biosynthesis and swarming motility (Zhao *et al.*, 2009b). Two major regulators in amylovoran biosynthesis and swarming motility have been identified, including EnvZ/OmpR and GrrSA (Zhao *et al.*, 2009b). In this study, our results further demonstrated that EnvZ/OmpR and GrrSA systems synergistically regulate *E. amylovora* amylovoran and levan production. Moreover, the two systems synergistically regulate *E. amylovora ams* and T3SS gene expression, but antagonistically regulate swarming motility.

A network of regulatory proteins has been reported to be involved in amylovoran biosynthesis, including global regulator H-NS and Rcs system (Mukherjee *et al.*, 1996, Wang *et al.*, 2009). We demonstrated, for the first time, that the two triple mutants, *ERS* and *ERA* produced higher amount of amylovoran compared with WT and *envZ, ompR, E/R, grrA, grrS* and

grrS/A single and double mutants. These data was verified by *in vitro* promoter activity assay and *in vitro* qRT-PCR.

Meanwhile, both EnvZ/OmpR and GrrSA TCSTs positively regulate levan production. Mutation in either system could partially reduce levan biosynthesis, and it appeared that GrrSA system has a dominant effect on levan production as compared with EnvZ/OmpR system since levan production of the two triple mutants was similar to those of *grrSA* single and double mutants.

On the other hand, EnvZ/OmpR and GrrSA systems antagonistically regulate *E. amylovora* swarming motility. In earlier report, we have identified that GrrSA and EnvZ/OmpR systems are negative and positive regulators of swarming motility, respectively. In this study, we further demonstrated that these two systems function oppositely in controlling the swarming ability of *E. amylovora.* The triple mutant *ERS* and *ERA* showed intermediate swarming ability.

Moreover, EnvZ/OmpR and GrrSA systems are involved in T3SS regulation. Our *in vitro* promoter activity and both *in vitro* and *in vivo* qRT-PCR results indicated that both systems negatively regulate *hrp*-T3SS genes expression. Similarly, the other two T3SS, PAI-2 and PAI-3, also seemed to be negatively regulated by EnvZ/OmpR and GrrSA systems. In addition, there is a synergistic effect between EnvZ/OmpR and GrrSA systems on T3SS gene expression. However, when qRT-PCR assay using mRNA isolated from immature pear fruits was performed, expression of *hrpL* gene was not changed. It is highly possible that *hrpL* mRNA level decreased after 18 hours *in vivo*.

Further study is needed to investigate the mechanism of EnvZ/OmpR and GrrSA in regulating amylovoran, swarming motility and T3SS gene expression. One possibility is through the RNA-binding protein RsmA & small regulatory RNA *rsmB* system. Systematic studies of the Rsm system in γ-proteobacteria have revealed that this system play an important role in controlling a large variety of physiological processes including central carbon metabolism, motility, virulence and biofilm formation, by binding to conserved sequences in its target gene mRNAs and altering their translation and/or turnover (Romeo *et al.*, 2012). It is likely that GrrSA system in *E. amylovora* may regulate EPS production and T3SS through the RsmA/*rsmB* system.

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